STUDIES OF RARE GENETIC VARIANTS IN AGE-RELATED MACULAR DEGENERATION

MAARTJE GEERLINGS

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Maartje J. Geerlings

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Promotoren:
Prof. dr. Anneke I. den Hollander
Prof. dr. Carel B. Hoyng

Copromotor:
Dr. Eiko K. de Jong

Manuscriptcommissie:
Prof. dr. Irma Joosten (voorzitter)
Prof. dr. Frans P.M. Cremers
Dr. Simon J. Clark (University of Manchester, Verenigd Koninkrijk)

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ABBREVIATIONS

Commonly used abbreviation

aHUS	atypical hemolytic uremic syndrome
AMD	age-related macular degeneration
AP	alternative pathway
ARPE-19	acute retinal pigment epithelial-19 cells
BM	Bruch's membrane
BMI	body mass index
C3G	C3 glomerulopathy
CA	cofactor activity
CADD	combined annotation dependent depletion
CFA	cofactor activity
CIRCL	Cologne image reading center and laboratory
CMC	combined multivariate and collapsing (burden test)
CNV	choroidal neovascularization
CP	classical pathway
DAA	decay-accelerating activity
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EMD	electron microscopy databank
ETDRS	Early Treatment Diabetic Retinopathy Study
EUGENDA	European Genetic Database
ExAC	Exome Aggregation Consortium
GA	geographic atrophy
GAG	glycosaminoglycan
GATK	genome analysis toolkit
GCL	ganglion cell layer
GWAS	genome-wide association study
HWE	Hardy Weinberg equilibrium
IAMDGC	International Age-related Macular Degeneration Genomics Consortium
IBD	identity by descent
IN	inner nuclear layer
IOP	intraocular pressure
IPL	inner plexiform layer
IS	inner segments
LD	linkage disequilibrium
LDH	lactate dehydrogenase
LP	lectin pathway
MAC	membrane attack complex

MAC-IP	membrane attack complex inhibitory protein
MAF	minor allele frequency
MDS	multi-dimensional scaling
NA	not applicable / not available
NFL	nerve fiber layer
NGS	next-generation sequencing
OCT	optical coherence tomography
ONL	outer nuclear layer
OPL	outer plexiform layer
OR	odds-ratio
OS	outer segments
PCR	polymerase chain reaction
PDB	protein databank
PhyloP	phylogenetic p-values
PolyPhen2	polymorphism phenotyping version 2
RCA	regulators of complement activation
RPE	retinal pigment epithelium
SD	standard deviation
SIFT	sorting intolerant from tolerant
SKAT	sequence kernel association (burden) test
SNP	single nucleotide polymorphism
SNV	single nucleotide variant
sTCC	soluble terminal complement complex
TCC	terminal complement complex
USA	United States of America
VQSR	variant-quality score recalibration
VT	variable threshold (burden test)
WES	whole-exome sequencing
WGS	whole-genome sequencing

Genes and proteins

ARMS2	age-related maculopathy susceptibility 2
BEST1	bestrophin 1
C2	complement component 2
C3	complement component 3
C4BP	C4b-binding protein
C5	complement component 5
C9	complement component 9
CD46 / MCP	membrane cofactor protein
CD55 / DAF	decay-accelerating factor
CFB / FB	complement factor B
CFD / FD	complement factor D
CFH / FH	complement factor H
CFHR	complement factor H related
CFI / FI	complement factor I
COL8A1	collagen type VIII alpha 1 chain
CR1	complement receptor 1
CRP	c-reactive protein
CTNNA1	catenin alpha 1
ELOVL4	elongation of very long chain fatty acid 4
FBN2	fibrillin 2
FP	factor properdin
FSCN2	fascin actin-bundling protein 2
HMCN1	hemicentin 1
IMPG1	interphotoreceptor matrix proteoglycan 1
OTX2	orthodenticle homeobox 2
PRDM13	PR domain containing 13
PROM1	prominin 1
PRPH2	peripherin 2
RP1L1	retinitis pigmentosa 1 like 1
SLC16A8	solute carrier family 16 member 8
THBD	thrombomodulin
TIMP3	tissue inhibitor of metalloproteinases 3
UBE3D	ubiquitin protein ligase E3D
VEGF	vascular endothelial growth factor
VTN	vitronectin

Protein domains

ANA	anaphylatoxin
CCP	complement control protein
COL1	triple-helical region 1
CTC	C-terminal complement
CUB	complement protein subcomponents C1r/C1s
CYT	cytoplasmic anchor
EGF-like	epidermal growth factor - like
FIMAC	factor I membrane attack complex
LAMB1	laminin beta-1
LDLr1 / LDLr2	low-density lipoprotein receptor 1 and 2
LDLRA	low-density lipoprotein receptor class A
LNK	hydrophobic linker
MACPF	membrane attack complex perforin
MG	macroglobulin
NC1 / NC2	non-collagenous 1 and 2
SCR	short consensus repeats
SP	serine protease
SRCR	scavenger receptor cysteine-rich
TED	thioester domain
TM	transmembrane
TSP1	thrombospondin type-1

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1A

INTRODUCTION

ANATOMY OF THE HUMAN EYE

Light enters the eye through the cornea, pupil and lens to ultimately focus on the central retina at the back of the eye. The retina is the neural portion of the eye with the ability to convert light entering the eye into biochemical signals. The retina is comprised of various cell layers including the ganglion cell layer, inner nuclear layer (containing the cell bodies of the amacrine, bipolar and horizontal cells), and outer nuclear layer (containing the cell bodies of the photoreceptors) which together are involved in processing light signals (**Figure 1**). The actual conversion of light into biochemical signals occurs in the outer segments of the photoreceptors, through a series of reactions known as the phototransduction cascade. The signals are then transferred to the optic nerve to be translated into visual perception by the brain.[1,2]

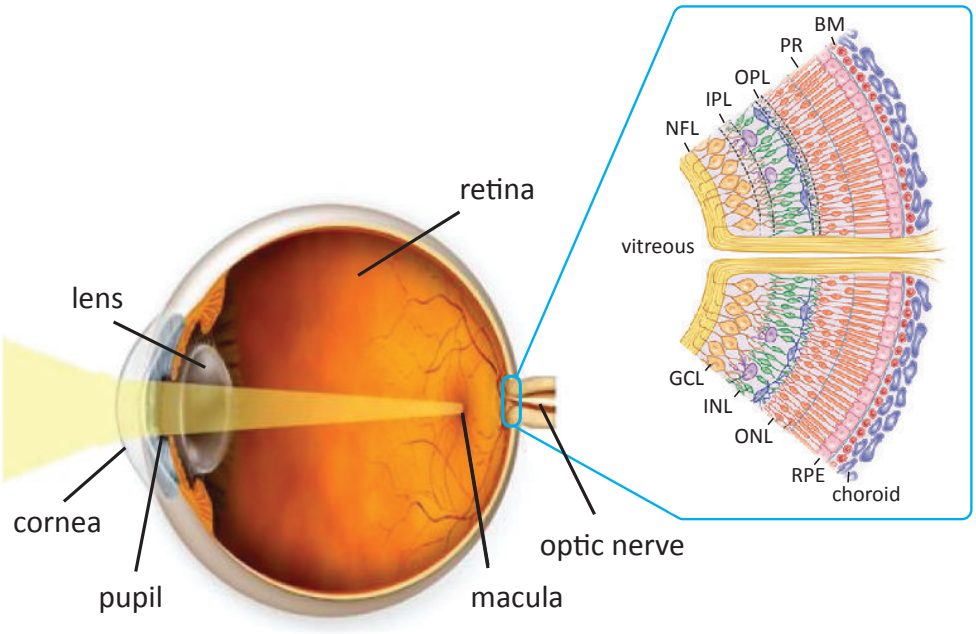

Figure 1: Anatomy of the human eye. Cross section of the retinal cell layers from anterior to posterior: vitreous, nerve fiber layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), photoreceptors (PR), retinal pigment epithelium (RPE), Bruch's membrane (BM) and choroid. Adapted from [3,4].

There are two types of photoreceptors in the retina, namely rod and cone cells. Rods are extremely sensitive and are activated under low luminance conditions. With increased illumination, cones participate more dominantly than rods in determining vision enabling color detection and high resolution vision. On average, the human retina contains 20 times more

rods than cones, however modern-day vision is predominantly regulated by the cone system, mediating high visual acuity under bright light conditions.

The macula is a region located centrally in the retina. The macula is a pigmented area, appearing yellow, due to the presence of lutein, zeaxanthin and xanthophyll pigments. The macular pigments absorb the harmful short wavelengths of visible light, acting as natural sunglasses. In addition, lutein and zeaxanthin function as antioxidants, which is particularly helpful in an oxygen-rich region like the macula, as it is extremely vulnerable to oxidative damage.[5] The center of the macula, known as the fovea, is a highly specialized region packed with cones.[2] The retinal pigment epithelium (RPE) is a melanin-containing cell layer which performs essential roles in maintaining and supporting photoreceptors. The main functions of the RPE include recycling of the photopigment chromophore 11 cis-retinal for the phototransduction cascade, protection against photo-oxidation by absorbing light, and phagocytosing the outer segment discs.[2] The RPE and Bruch's membrane (BM) complex form the outer blood-retina barrier, which regulates the rapid exchange of waste products, oxygen and nutrients between photoreceptors and the choroidal vasculature, functioning as a protective barrier and maintaining retinal homeostasis.[6]

AGE-RELATED MACULAR DEGENERATION

Age-related macular degeneration (AMD) is the leading cause of irreversible vision loss among elderly individuals, accounting for 8.7% of blindness worldwide. The disease is most prevalent in populations of European ancestry with approximately 1-3% of the total population suffering from an advanced form of AMD.[7-9] Globally, the total number of patients with any type of AMD is expected to increase to 288 million affected individuals in 2040.[9] Patients experience blurring of their central visual field or images may appear as distorted (metamorphopsia), in addition to difficulties with seeing colors and fine details. Over time, the central field may become obscured and central vision loss is experienced (scotoma) as the disease progresses.[10]

Clinical presentation

The early clinical signs of AMD are the appearance of yellowish deposits, called drusen, between the RPE and BM. Drusen can be classified according to type, morphology and size. Soft drusen range in size from small (≤63μm in diameter) to large (≥125μm) with diffuse borders. The increase in size and number of soft drusen is a sign of progressing AMD. Hard drusen are smaller in diameter and have more defined borders. Hard drusen are a common sign of aging and an independent risk factor for vision loss in AMD.[11] When soft and hard drusen undergo calcification they are referred to as crystalline drusen, due to their glistening appearance.[12,13]

Cuticular drusen (also known as basal laminar drusen) refer to a specific drusen phenotype characterized by a large number of small (hard) drusen scattered throughout the retina.[14]

Presentation on funduscopy

An ophthalmologist can examine the retina to evaluate the nature of the disease, for example by capturing a photograph of the inner lining of the eye through the pupil. A fundus photograph visualizes the main structures of the retina like the central (macula) and peripheral retina, the optic nerve head (or the optic disc), and the retinal vessels (**Figure 2**). In patients with an early form of AMD the yellowish drusen, clustering around the macula, are clearly visible on fundus photographs.[15]

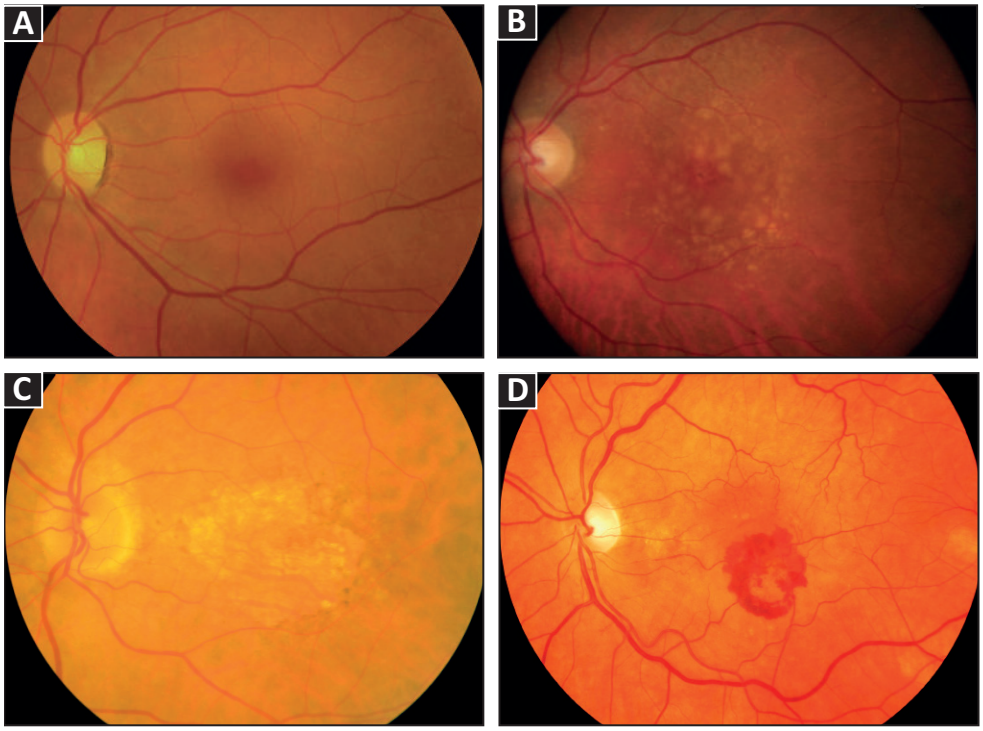

Figure 2: Fundus photograph of a healthy retina (A) and different stages of AMD (B-D). The darker patch in the center of the image is the macula, and the optic disk is located on the left. B) Intermediate AMD with extensive intermediate to large drusen primarily located in the macula. C) Advanced AMD with central geographic atrophy surrounded by soft drusen. D) Advanced AMD with choroidal neovascularization with central hemorrhage.

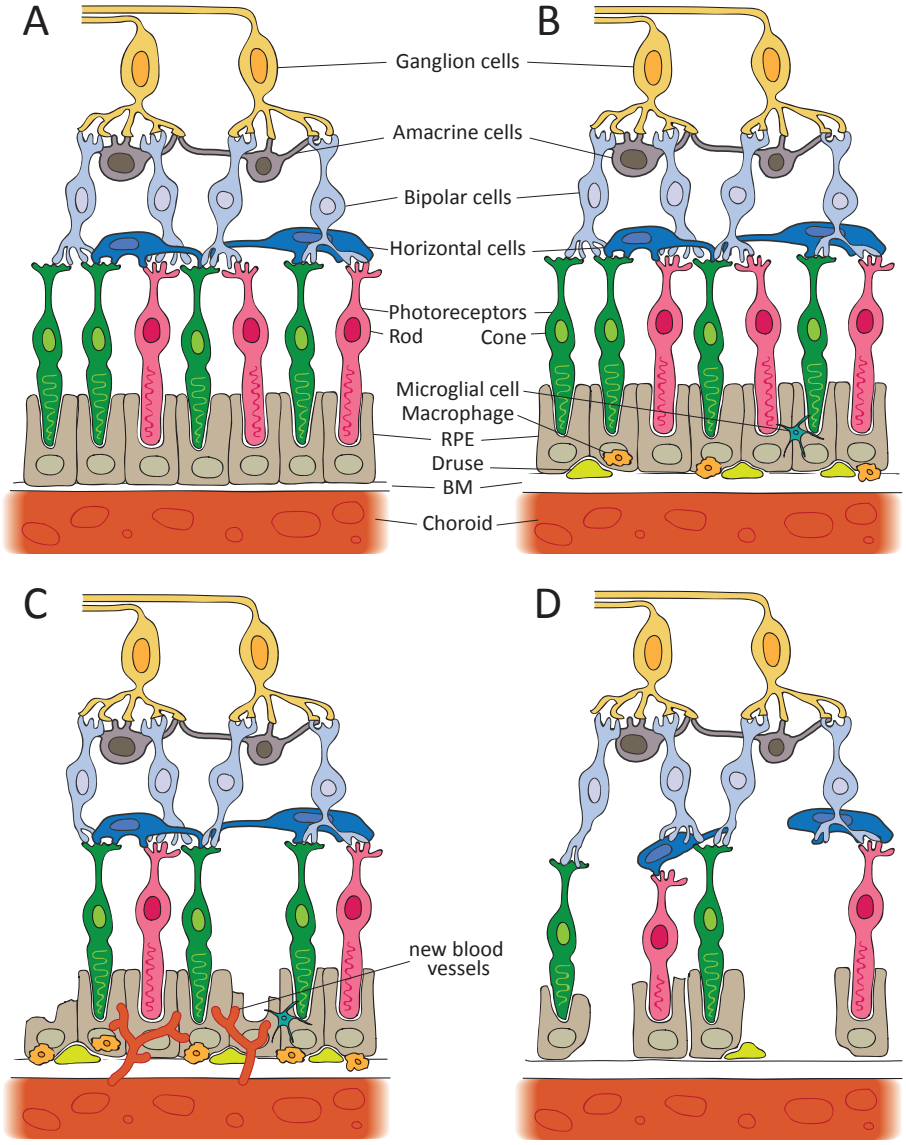

Figure 3: Graphical representation of the retinal anatomy in normal and various AMD stages. (A) Normal retinal architecture focused at the macula comprised of various cell layers from anterior (top) to posterior (bottom). (B) As the disease progresses Bruch's membrane (BM) thickens and drusen form between the BM and the retinal pigment epithelium (RPE), attracting immune cells like macrophages and microglia cells. Eventually, the disease can progress in one of two late forms: wet (C) and dry (D) AMD. Wet AMD is characterized by the invasion of abnormal and leaky blood vessels and an accumulation of macrophages. In dry AMD general degeneration of the RPE and photoreceptors is observed.

Drusen composition

Drusen contain a high variety of components, including proteins, lipids, cholesterol, and cellular fragments of the RPE. The main proteins found in drusen include: immunoglobulin and components of the complement pathway (complement receptor 1, C3, C5, membrane cofactor protein, and terminal complex C5b-9), acute inflammation response (amyloid beta) and immune response modulating proteins such as vitronectin, clusterin, apolipoprotein E, fibrinogen, HLA-DR and enzymes like factor X and prothrombin.[16-22]

AMD PATHOLOGY AND STAGING

In early stages of the disease drusen number is limited and does not affect visual function. As the number of drusen increases, or pigmentary changes are apparent in the retina due to the degeneration of RPE cells, the disease progresses from early to intermediate AMD[23,24] (**Figure 3B)**. Eventually the disease can progress to late AMD, which exists in two distinguished forms: neovascular AMD, known as wet AMD, or geographic atrophy, known as advanced dry AMD. The neovascular form is characterized by infiltration of abnormal blood vessels from the choroidal vasculature into the retina. These newly formed vessels are fragile and break easily, leaking blood in the retina and leading to sudden vision loss (**Figure 3C**). The second form, geographic atrophy, is the result of gradual degeneration of RPE and photoreceptors cells as well as the constriction of choroidal blood vessels.[23,24] (**Figure 3D**).

Although neovascularization occurs in only 15-20% of cases, it is responsible for the vast majority of vision loss caused by AMD. Intraocular injections of drugs targeting vascular endothelial growth factor, one of the central molecules in neovascularization, have proven to be very successful in neovascular AMD.[25] However, no treatment is available for patients who have early, intermediate or geographic atrophy AMD. Furthermore there are currently no effective means of preventing progression of early to advanced stages.[26,27]

AMD IS A MULTIFACTORIAL DISORDER

Multifactorial and 'complex' are commonly used synonyms, meaning that the disease clearly has a heritable component, but is also partially influenced by environmental and lifestyle factors. A variety of non-genetic factors has been identified, of which aging, ethnicity, dietary habits and cigarette smoke are consistently associated with AMD.[7,9,15,28]

Environmental factors

Age is the most dominant risk factor for AMD; as individuals age the risk for developing AMD increases as well. Within the age group 55 to 64 years of age, advanced AMD is present in 0.2% of the population, while this number rises to 13% in the group of 85 years and older.[29]

Cigarette smoke is the most important modifiable risk factor and has been associated with a two-to-three fold risk of developing AMD. A clear dose-response effect is observed, where increased cigarette consumption leads to an increased risk of developing AMD. Moreover, former smokers still have modest risk of developing AMD compared to non-smokers.[7,30] Cigarettes contain numerous toxins which act through different biochemical mechanisms and may result in oxidative damage, vascular changes, and inflammation.

Oxidative stress affects the RPE and especially the macula. The macula is extremely vulnerable for oxidative stress due to its high metabolic activity, high oxygen demand and polyunsaturated fatty acid content which is prone to oxidation.[30] Dietary intake of antioxidants and zinc reduce the risk of developing AMD in elderly individuals.[31,32] High doses of oral antioxidants (vitamin C, vitamin E, and carotenoids lutein and zeaxanthin), in addition to zinc, reduce AMD progression. It was shown that these supplements were able to reduce AMD progression by approximately 25% over 5 years.[27,31,33,34]

Heritability

Evidence for a strong genetic component in AMD was established using twin and family studies. Twin studies observed a high concordance of AMD between monozygotic pairs, even double compared to dizygotic pairs, and estimated the heritability of AMD to be as high as 46-71%.[35-37] Family studies noted a higher prevalence of AMD characteristics and an earlier onset of disease symptoms among relatives of patients compared to control families.[38-41]

GENETIC ANALYSES

AMD is a very heterogeneous disorder with a variety of genes and pathways implicated in the disease pathogenesis. Identification of disease-causing variants can be done using techniques like genome-wide association study (GWAS) and next-generation sequencing (NGS).

Genome-wide association studies

GWASes are hypothesis-free approaches that use genetic variation across the genomes of thousands of individuals to search for genetic influences on traits. The success of GWASes is based on the common-disease common-variant hypothesis that postulates that common diseases, like AMD, can largely be explained by common variants found in more than 1-5%

of the population.[42,43] Using an array-based platform, on which several hundred thousands of single nucleotide changes can be captured, common variants with small to large effect sizes can be detected. In AMD, GWASes and subsequent meta-analyses have been exceptionally successful, which is due to the relatively large effect sizes of common variants at two AMD loci *(CFH* and *ARMS2/HTRA1)*. However, the majority of variants have an odds ratio (OR) between 0.7 and 1.5.[44-48] These variants collectively account for 15-65% of the genomic heritability, suggesting that other genetic factors must be involved in the disease etiology.[47]

The common-disease rare-variant hypothesis proposes that rare variants, or specifically multiple risk alleles each of which is individually rare, may explain the heritability.[49,50] Rare variants, found in <1% of the population, are by definition found in only a minority of individuals. Compared to common variants, these rare variants may have more obvious functional consequences as they are enriched in genomic regions that are evolutionary conserved, and are often predicted to affect protein function.[51,52] GWAS arrays are inefficient in tagging rare variants due to the low correlation between the variants, as rare variants are often private and in low linkage disequilibrium. However, arrays can be designed to capture rare genetic variants directly. The most recent GWAS in AMD used an array enriched with rare variants to uncover 52 variants, of which 45 were common, that could be grouped into four main pathways: (1) complement system, (2) high-density lipoprotein metabolism (3), angiogenesis, and (4) extracellular matrix remodeling. In addition, seven rare variants were identified which had an OR between 1.5 and 47.6, and were all located in or near genes of the complement system. In addition, a burden of rare genotyped variants was found for the *CFH, CFI, SLC16A8* and *TIMP3 genes, meaning that rare variants in these genes were collectively observed more frequently* in AMD cases than in controls.[47,48] It should be noted that the rare variants in these genes were manually enriched on the array. Extremely rare or novel rare variants will normally not be detected using GWAS but will require a broader detection of the gene as provided in next-generation sequencing (NGS).

Next-generation sequencing

In 1977, a method to sequence DNA by using chain termination was published by Fred Sanger.[53] Sanger sequencing allows for the sequential analysis of individual DNA sequences, limiting the ability to rapidly and affordably reading large parts of the genome. In 2005, however, a new sequencing approach was introduced in which the sequencing process was performed in a massive parallel fashion.[54] This nascence of massive parallel sequencing, also termed next generation sequencing, has dramatically changed our ability to read DNA, and nowadays we can reliably, rapidly and affordably sequence many or even all genes of a genome, or even the entire genome of a human being. The total amount of sequencing data is growing on a logarithmic scale, as it has been estimated that the amount of sequencing data is doubling approximately every seven months beyond the capacity to process and store the data.[55,56]

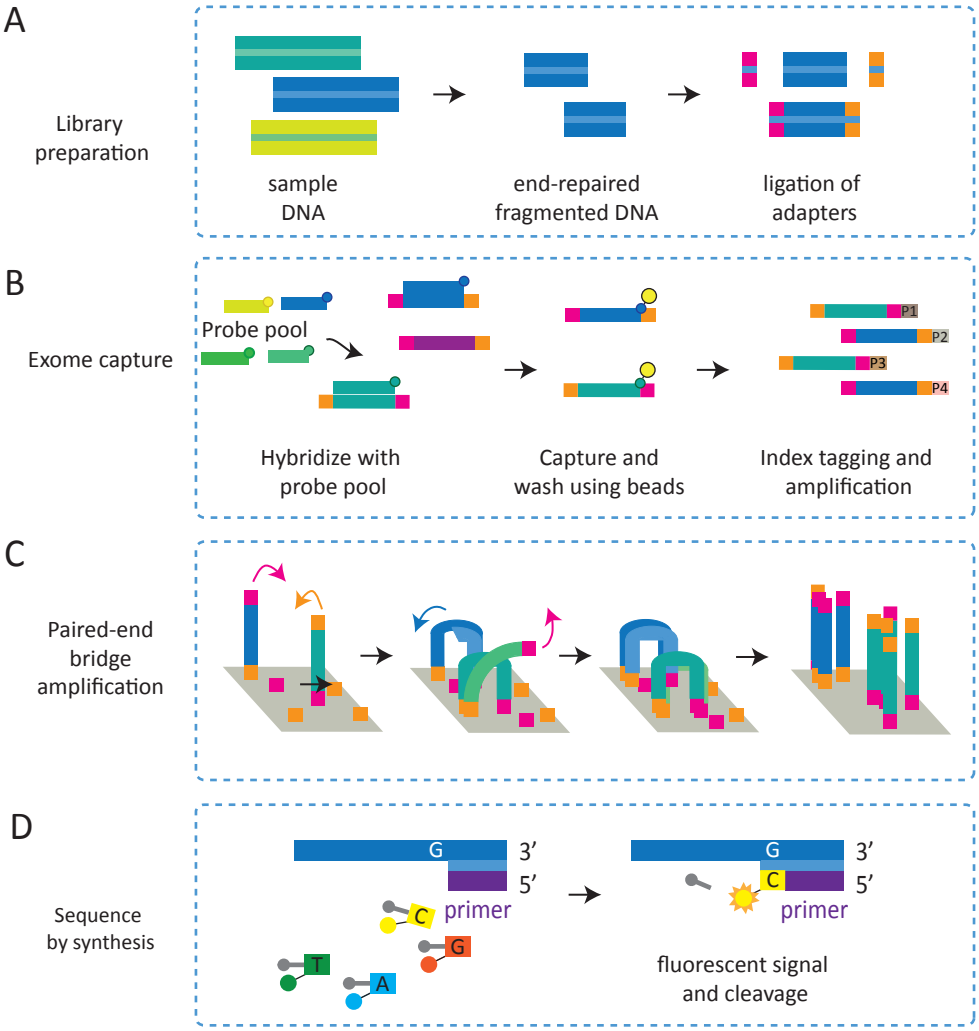

Figure 4: Example of a whole exome sequencing pipeline. The DNA is cut into smaller pieces and equipped with adapter sequences. Exome capture selects specific regions of the genome and redundant fragments are washed away. Index tags are added for recognition and the fragments are amplified. The fragments bind to a flow cell for paired-end clustered bridge amplification. The fragments are read using sequencing by synthesis: a step-by-step incorporation of fluorescent nucleotides. The fluorescent signal is read at each cluster and recorded until the reaction is complete.[59,60] Adapted from [61].

Whole-exome sequencing (WES) captures and sequences the entire coding part of our genome, all of the exons of all of our genes. This exome comprises approximately 1% of the entire human genome. Whole-genome sequencing (WGS) encompasses the complete genome, including intronic and intergenic regions.[57,58] WES relies on the principle of simultaneously capturing thousands of fluorescent signals. In short, DNA samples are subjected to several

preparatory steps in a process called library preparation. The DNA is randomly fragmented into smaller pieces followed by end-repair and ligation of adapter sequences. Specific regions of the DNA are captured, amplified by polymerase chain reaction and clustered together with unique barcodes. Sequencing is performed by repeating cycles, in which fluorescently labeled nucleotides terminate the sequence, and the signal is captured (**Figure 4**).[59,60]

Output of a NGS reaction is an uninterrupted series of nucleotides ('sequencing reads'), and after aligning each of these reads to the reference genome we can calculate how often a specific nucleotide is sequenced (termed 'sequence coverage'). Depending on the used capture kit, WES identifies between 20 and 50 thousand variants per sequenced exome. To reduce false-positives the data is filtered for standard quality criteria like minimum coverage per region or consistent call rate in more than 5% of the samples. Vigorous filtering of the data or comparing the variant load between case and control groups is required to uncover variants playing a potential role in disease pathogenesis.

Analysis of NGS data assuming Mendelian inheritance

Mendelian traits are caused by variation in a single gene and recognizable by a classic inheritance pattern. Sometimes multifactorial diseases can behave as Mendelian traits, showing clustering in families and presenting with a more severe phenotype or an earlier age of onset. Assuming monogenic inheritance in such families, it is a matter of reducing the number of variants identified by NGS until a limit set of variants remains. First, variants can be selected that are shared among all affected individual family members. Next, variants can be selected that alter protein function, as these are potential disease-causing variants (splice site or non-synonymous variants). Subsequently, public databases like 1000genomes,[62] The Exome Aggregation Consortium (ExAC),[63] or Exome Variant Server[64] can be consulted to filter out common variants.[58] The remaining variants can be prioritized based on specific genes of interest (in a candidate gene study) or predicted to be pathogenic by *in-silico* prediction.[65-67] A number of studies used this approach to identify rare variants in families affected by AMD.[68-74] Rare genetic variants reported in AMD families are reviewed in more detail in **Chapter 1B: The complement system in age-related macular degeneration**.

Analysis of NGS data using association analyses

Complex traits, like AMD, often do not follow the predictable patterns of Mendelian inheritance and other approaches are required for the analysis of NGS data. NGS strategies have only recently been adopted to identify rare genetic variants in complex diseases.[75,76] The first successful report of WGS in AMD was in 2013 in a large population-based cohort[77] from Iceland. Two more studies used WES to identify risk variants in an AMD case-control cohort.[78,79] Genetic variants reported in AMD are reviewed in more detail in **Chapter 1B: The complement system in age-related macular degeneration**.

NGS can be applied to case-control cohorts, followed by association analyses to identify rare variants that are associated with the disease. The initial analysis that can be performed is a single-variant analysis, in which the association of individual rare variants with the disease is tested. However, the bottleneck with rare variants detection is the large sample size required compared to common variants, especially if the effect size of the rare variants is small. Therefore, case-control studies are often underpowered to detect single rare variant associations.[80] Instead of single variant analyses, aggregation tests (also called burden tests) evaluate the cumulative effect of multiple genetic variants in a single gene, region or pathway. Variant data is grouped and association analyses in cases and controls is performed.

There are two types of burden tests, (1) variants in a region are assumed to have the same direction of effect (damaging or protective), or (2) variants in a region are allowed to have opposite effects.[81] The first test is simpler as it collapses variants, scores them, and tests for the association between the score and a trait. Examples include the Combined Multivariate and Collapsing (CMC) or Variable Threshold (VT) burden tests.[82,83] The second test, known as a variance component test, assesses variants distribution also when the consequences are in opposite directions, like the Sequence Kernel Association Test (SKAT)[84].

THE COMPLEMENT SYSTEM IN AMD

Before any specific gene or biological pathway had been conclusively linked to AMD, studies into the molecular constituents of drusen suggested that AMD may have an immunological component. These theories were based on proteins found in drusen which are involved in inflammation and/or other immune-associated responses, including components of the complement system.[17,19,21,22]

The alternative pathway of the complement system became a major focus in AMD research after the identification of risk variant in *CFH* p.Tyr402His in 2005.[85] Ever since, genetic variants have been identified in the alternative pathway of the complement system, which plays an important role in AMD pathogenesis.[47,48,86-94] In the most recent GWAS, twelve of the 45 identified common variants and all rare variants (all seven) resided in or near a gene of the complement system: *CFH, CFI, C3, C2/CFB, C9* and *VTN*.[48]

There are several theories on how complement activation contributes to retinal damage. Some theories focus on functional loss of RPE triggered by external factors (like smoking and aging), which accelerate complement activation. Others focus on local activation of the complement system by drusen. Genetic variations add to these scenarios as they may lead to hyperactivation of the complement system or obstruct proteins from properly preventing

unwanted complement attack.[95-97] Continuous low levels of complement activation are beneficial for the removal of cellular debris. However, decades of low-grade inflammation may lead to tissue damage.[98] In AMD, increased levels of systemic complement activation are observed in serum or plasma. The activation markers Ba, Bb, C3a C3d, C5a and regulators FB and FD are higher in patients with AMD compared to (age-matched) controls in addition to decreased FH levels.[99-103] Complement activation products were also found locally in aqueous humor, vitreous and BM in (postmortem) eyes of AMD patients [104-106]. Furthermore, genetic variants in complement genes linked to AMD have a direct effect of systemic complement activation.[99-102,107] In addition, AMD disease stage, age, BMI and smoking individually influence complement levels as well.[99-102,107-109]

The common AMD-associated variant *CFH* p.Tyr402His, located in SCR7, results in reduced binding of FH to the BM and inner choroid which could explain the AMD phenotype.[110] Moreover, the CCP6-8 mediates recruitment of FH on the RPE and BM in the eye, while the CCP19-20 region is involved in tissue-specific binding with the glomerular basement membrane in the kidney.[110] For *CFI*, rare AMD-associated genetic variants in the *CFI* gene result in reduced systemic FI levels and consequently lower the regulatory activity of the alternative pathway.[91,92] Knowledge is lacking regarding the effects of genetic alterations on complement protein C9 and MAC formation, in relation to AMD. However, MAC deposits have been identified within drusen and in BM,[28] and sublytic MAC induces an inflammatory response in cultured RPE cells, suggesting local inflammation.[111]

THE COMPLEMENT SYSTEM: THE ALTERNATIVE PATHWAY

The complement system is part of the innate immune system and tight regulation of this system is needed to protect the body's own cells from tissue damage. The complement system accomplishes its goal, self-defense against invading pathogens, by (1) attacking the microbial membrane by generating convertases, (2) generating millions of defense molecules at great speed and bulk, and (3) creating local inflammatory sites.[21,112-114]

There are three activation pathways of the complement system. The lectin pathway, activated by mannose-binding lectin, the classical pathway activated by antibody-antigen complexes, and the alternative pathway which is activated by spontaneous hydrolysis. Auto-activation of the alternative pathway is unique as it relies on the hydrolyzing of labile C3 thioester bonds, in a process known as tickover. Upon activation C3a is cleaved off C3 forming C3b whereby the exposed thioester bonds have the ability to form covalent bonds. These covalent bonds bind freely to the surfaces of cells and of microbes.[115]

The C3 and C5 convertases catalyze further steps in the cascade by (1) initializing a feedback loop, and (2) starting the breakdown cycle. In the breakdown cycle, surface-bound C3b can be inactivated by serine protease factor I (FI) into its inactive form iC3b, which requires the help of cofactors (**details provided below in Chapter 1.6.3. Factor I**). Further steps of the breakdown cycle are illustrated and explained in **Figure 5**. In the feedback loop, stabilized C3 convertase (C3bBb) can generate numerous C3b molecules. The additionally generated C3b aid in formation of the C5 convertase and continued activation of the alternative pathway.[112-114,116] (**Figure 5**).

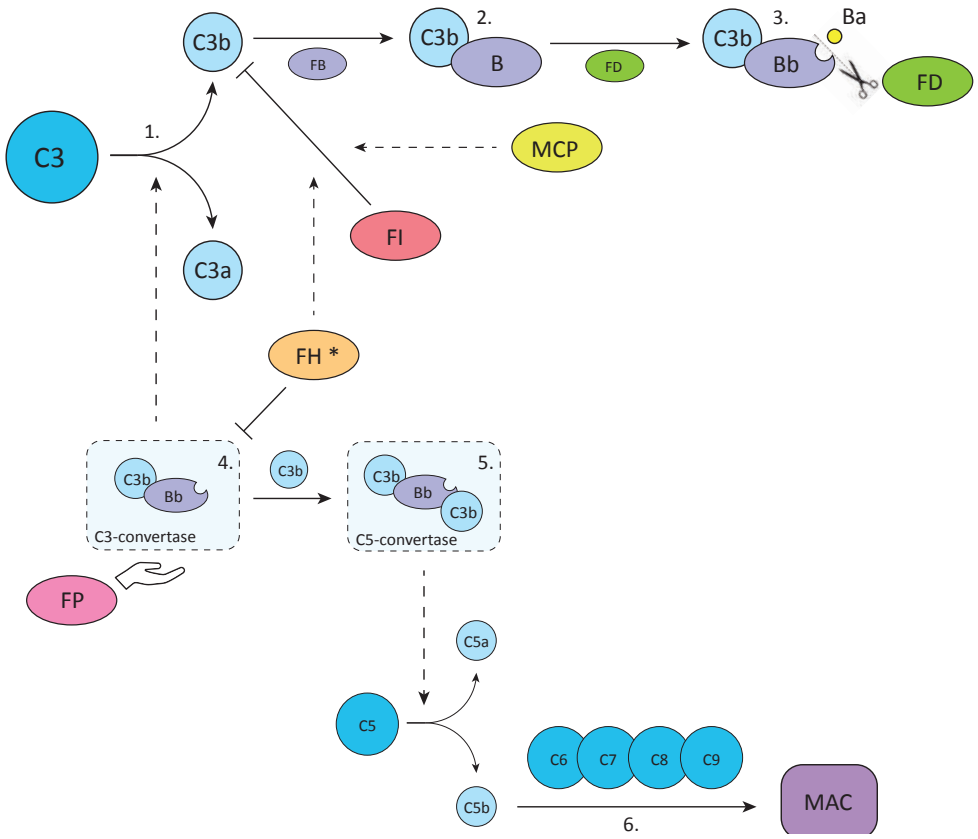

Figure 5: The alternative pathway of the complement system. 1. The alternative pathway is activated the moment C3 is spontaneously hydrolysed. 2. C3 can swiftly amplify by employing proteases factor B (FB) 3. Factor D (FD) together with stabilizing protein properdin (FP) forms the C3 convertase (C3bBb). 4. The C3-convertase can, through an efficient feedback loop, generate large amounts of C3b to opsonize pathogens or can bind to additional C3b to form the C5-convertase (C3bBbC3b). 5. The C5-covertase cleaves C5 into C5a and C5b. 6. C5b initiates the membrane attack complex (MAC; C5b-9), by binding sequentially to C6, C7, C8 and multiple C9 molecules. *Inhibition of the C3-convertase is achieved by regulators like Factor H (FH) and decay accelerating factor (DAF). **Factor I (FI) can digest C3b through cofactor mediated cleavage with FH or Membrane Cofactor of Proteolysis (MCP; CD46). Figure adapted from [117].

C3

C3 is the central component of the complement system. C3 interacts with a wide range of complement factors, including proteases, receptors and regulators but also viral and bacterial proteins through distinct binding sites. Anaphylatoxin C3a modulates inflammation and possesses antimicrobial activity. C3b has the ability to bind to the cell surface of invading pathogens via the exposed thioester bond.[118]

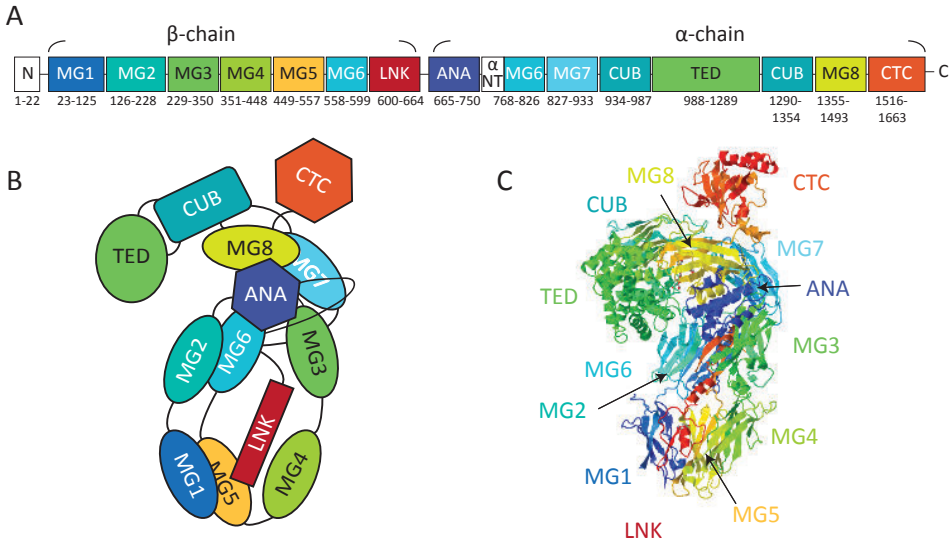

Figure 6: Structure of C3. (A) Schematic structure of C3 divided in the β and α-chain with amino acid numbering corresponding to the mature protein. (B) A graphical representation of the domain arrangement relative orientations in the crystal structure as shown in C. (C) Superposition of C3 with individual domains, based on crystallized structure submitted in the protein data bank (PDB) accession number 2A73.[118]

Structurally, C3 consists of an α-chain and β-chain linked by a disulfide bridge. The β-chain starts the formation of a ring-like structure with macroglobulin (MG) domains and a hydrophobic linker (LNK). The anaphylatoxin (ANA) domain, released as the C3a fragment. The remaining domains, piled on the ring, are CUB (complement C1r/C1s, Uegf, Bmp1) domain framing and holding the globular thioester-containing domain (TED; C3d), and an anchored C-terminal complement domain (CTC)(**Figure 6).**[118,119] In the formation of C3 to C3b, the β-chain remains largely unchanged whereas the α-chain is rearranged with the TED located at the other side of the MG ring. The repositioned CUB is involved in binding FB and FI cofactors.[120,121]

Factor H

Factor H (FH) is the main inhibitor of complement through binding to C3b and aiding FI in cofactor mediated cleavage. The FH protein is made up of 20 short consensus repeats (SCRs) connected by short linkers which fold into distinct three-dimensional structures named complement control protein (CCP) modules (**Figure 7**). While the 20 modules seem highly similar in appearance, there are several distinct functional regions. The first four CCPs (CCP1-4) form a C3b binding site by competing with FB, and in addition make C3b susceptible for cleavage by FI.[122] Regions CCP6-8 and CCP19-20 are similar in function, and can interact with glycans (like glycosaminoglycans (GAGs)), lipid peroxidation products, extracellular matrix proteins, apoptotic or necrotic cells and foreign bacteria.[123]

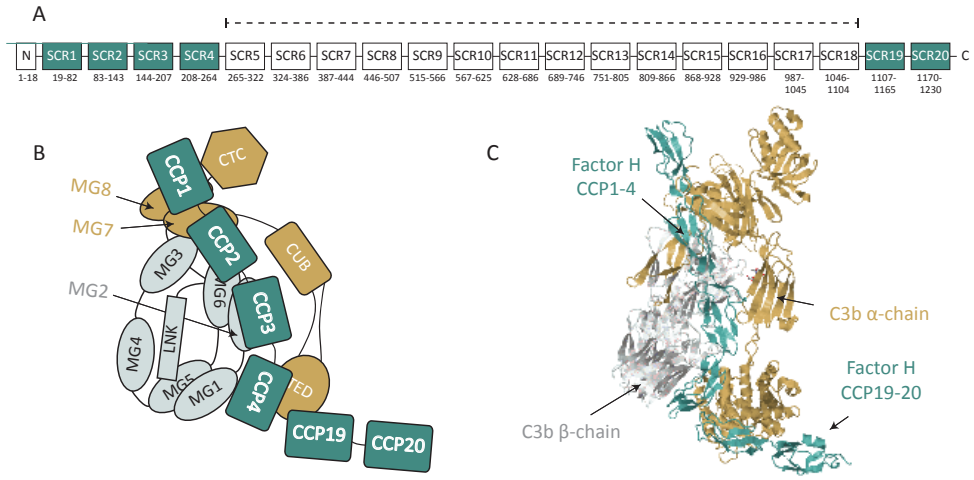

Figure 7: Structure of FH and interaction with C3b. (A) Schematic structure of FH with amino acid numbering corresponding to the mature protein with signaling peptide. The two major functional regions are colored green are functioning as binding sites for C3b/C3d, heparin/glycosaminoglycans and cell surfaces. There are no crystallized structures available for SCR5-18. (B) A representation of the domain arrangement partial FH 'miniFH' in interaction with C3b. MiniFH consists of CCP domains 1–4 and 19–20, connected by 12 glycine residues. (C) superposition of C3b-miniFH with C3b in gold (α-domain) and silver (β-domain) and miniFH consisting of CCP1-4 and 19-20 binding to C3b and C3d, respectively. Based on PDB 5O35.[122]

Factor I

Factor I (FI) is a serine protease critical for regulation of the complement system by cleaving C3b in the presence of one the cofactors. Known FI cofactors are membrane bound membrane cofactor protein (MCP; CD46), and complement receptor 1 (CR1; CD35), next to plasma components FH and C4b-binding protein (C4BP).[113] FI can degrade C3b and C4b in fluid phase

or when deposited on the cell surface. The FI protein circulates in the blood and is unique is its inhibitory function as it has no natural blocker.[113]

FI consists of a heavy and a light chain linked by a disulfide bridge. The heavy chain contains a FI membrane attack complex (FIMAC) domain, a SRCR domain (scavenger receptor cysteine-rich domain, also called CD5), low-density lipoprotein receptor 1 and 2 domains (LDLr1 and 2) and a small undefined region. The light chain of FI comprises a serine protease (SP) domain (**Figure 8**).[124-126] The working theory is that a cofactor binds C3b and forms a stable platform for FI to adhere to. Once the heavy chain of FI is fastened, remodeling of the protein promotes allosteric activation whereby activation sites become exposed.[127]

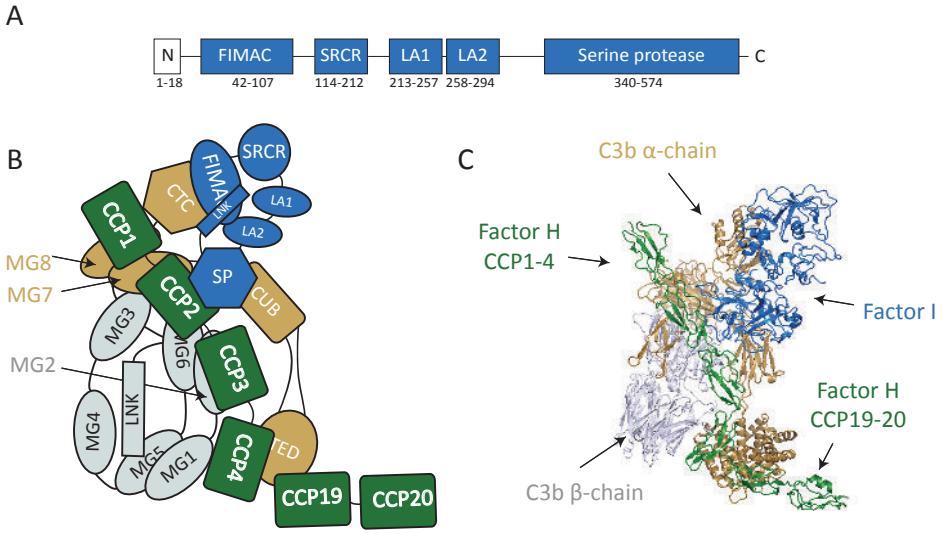

Figure 8: Structure of FI in interaction with FH and C3b. (A) Schematic structure of FI with amino acid numbering corresponding to the mature protein with signaling peptide. (B) A graphical representation C3b-miniFH-FI domain arrangement as their relative orientations in the crystal structure. MiniFH consists of CCP domains 1–4 and 19–20, connected by 12 glycine residues without the unknown structure of CCP5-18. (C) superposition of the C3b-miniFH-FI protein complex. FI binds in cofactor activity with C3b-miniFH at the CTC, MG2 and CUB domain of C3b in addition to CCP2-3 of FH. Based on crystallized structure PDB 5032 122, adapted in PyMOL.

Regulators of complement activation

The regulators of complement activation (RCA) protein/gene cluster controls complement activation. The family consists of decay-accelerating factor (DAF; DD55), CD46, CR1, FH and C4BP. The RCA members can act either through cofactor mediated cleavage or by decay-accelerating activity. Decay-accelerating activity disassociates the catalytic domain of C3 or C5 convertases, but only temporary as the convertases can reassemble.[95,114] Down-regulation of complement activation takes place on the cell surface and in fluid phase.

Complement regulators CR1, CD46, and DAF are expressed on cell membranes, on which they provide immediate protection to host cells. Soluble regulator FH stops complement activation in the fluid phase and differentiates between "self" or "non-self" cells and matrix material by binding host molecular patterns.[110,128]

Table 1: specification of regulators of complement activation proteins.

Protein	Function	Interaction	Pathway
CD46	CA	Surface (via transmembrane domain)	CP, LP and AP
CD55	DAA	Surface (via glycolipid anchor)	CP, LP and AP
CR1	CA/DAA	Surface (via transmembrane domain)	CP, LP and AP
FH	CA/DAA	Fluid (binds to C3b)	AP
C4BP	CA/DAA	Fluid (binds to C4b)	CP and LP

CA = cofactor activity; DAA = decay acceleration activity; CP = classical pathway; LP = lipid pathway; AP = alternative pathway.

C9 and the membrane attack complex

Cleavage of C5 into C5a and C5b is the first step of the formation of the terminal complement complex. C5b sequentially binds to C6, C7 and C8. Subsequently 22 copies of C9 (four in the tilt and 18 in the circular structure) are incorporated to form the membrane attack complex (MAC). This split-washer (open ring) formation can disrupt membrane integrity by penetrating the cell membrane (**Figure 9**). Vitronectin and clusterin can bind to the complex and yield a soluble complex which prevents C9 from penetrating the membrane. In addition, the complex will be unable to binding more C9 thereby effectively preventing pore formation. Furthermore, MAC-inhibitory protein (MAC-IP; CD59) located on host membranes can prevent C9 from polymerizing and MAC forming.[129-134]

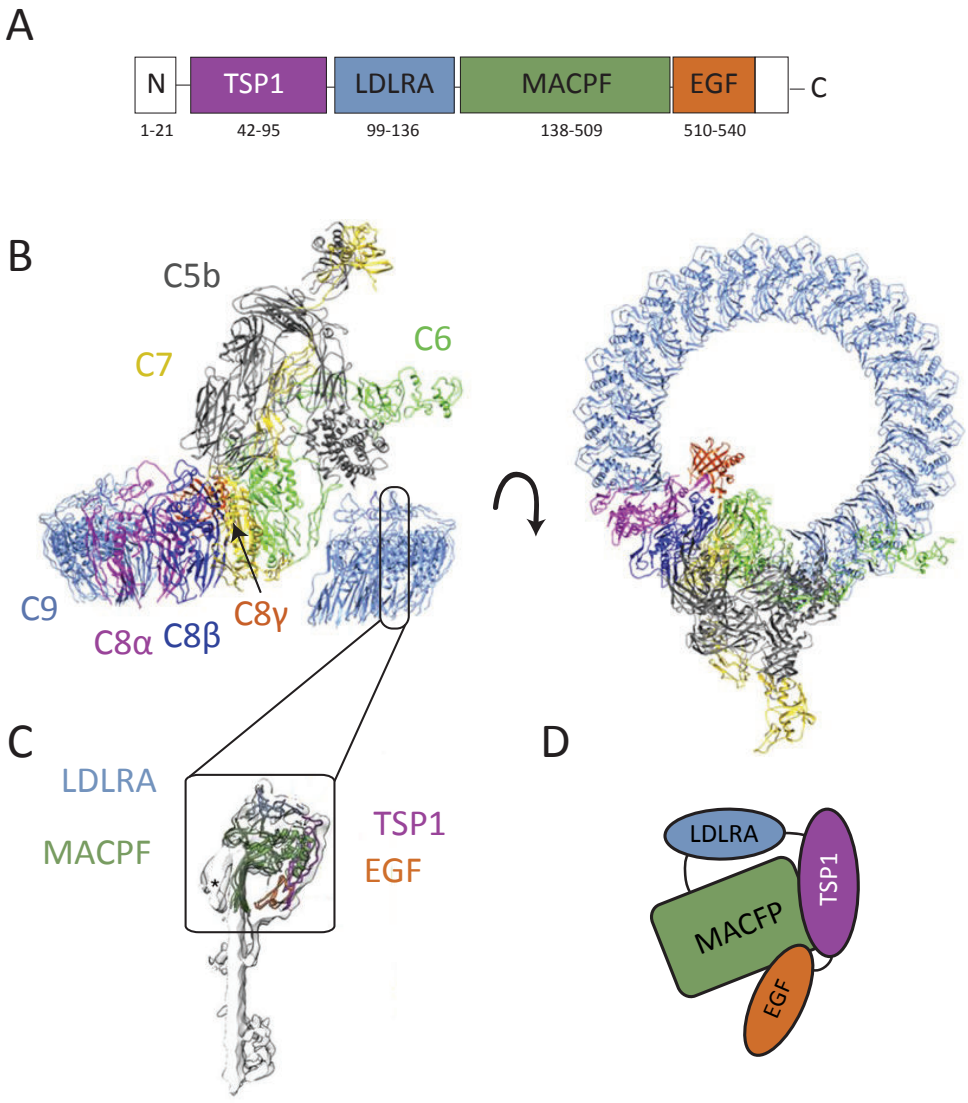

Figure 9: Structure of C9 and the membrane attack complex. (A) Schematic structure of C9 with amino acid numbering corresponding to the mature protein with signaling peptide. The protein consist of four domains: an N-terminal thrombospondin type-1 TSP domain (TSP1), a low-density lipoprotein receptor class A (LDLRA), MAC Perforin (MACPF) and a C-terminal epidermal growth factor (EGF-like) domain.[135] (B) Pseudo-atomic model of the membrane attack complex rim including the individually labeled complement proteins C5b to C9. Note: there is no crystallized structure available. (C) One C9 ribbon fitted into the reconstruction and the unmodeled β-helices below. (D) A graphical representation of the C9 ribbon with corresponding C9 domains. Adapted from [130] as submitted in Electron Microscopy Data Bank under accession numbers EMD 3134.

AIMS OF THIS THESIS AND CHAPTER INTRODUCTION

It was previously hypothesized that a common disease, like AMD, should be caused by common genetic variants. However, at the start of this thesis project, after large GWASes uncovered 19 loci, a large fraction of the heritability still remained unexplained.[47] Rare genetic variants, defined as genetic changes with a minor allele frequency below 1% in the population, can have large effects on disease and may account for the 'missing' heritability.

The aim of this thesis is to further elucidate the role of rare genetic variants in AMD pathogenesis. We hypothesize that a proportion of the missing heritability in AMD can be explained by rare highly penetrant variants. We aimed to identify novel genetic causes of AMD, and to understand their role in the disease mechanisms.

Chapter 1A: Introduction
This chapter provides the basic information needed to understand the current genetic en biochemical research in the ophthalmology field regarding AMD. An introduction is given on AMD, performing genetic analyses, and on the complement system.

Chapter 1B: The complement system in age-related macular degeneration
This chapter goes into depth on the common and rare genetic variants found in genes of the complement pathway, which have been found to play a role in the pathogenesis of AMD. It reviews all rare variants identified in AMD patients, and summarizes the functional consequences of rare genetic variation in complement genes.

Chapter 2: Rare genetic variants associated with development of age-related macular degeneration
Chapter 2 determines the contribution and segregation of rare genetic variants in the complement system in large families with AMD. Furthermore, clinical characteristics in carriers versus non-carriers of rare genetic variants in large families and in a case-control cohort are described.

Chapter 3: Rare variants in *CFH* and *CFI* result in decreased C3b degradation in patients with age-related macular degeneration
Chapter 3 uncovers novel rare genetic variants in complement genes previously found to be associated with AMD by using WES in AMD families. The chapter assesses the functional effect of rare genetic variants on levels of complement components in serum. C3b degradation ability was used as a marker of complement activation to assess the effect of different rare variants.

Chapter 4: Functional analysis of rare genetic variants in complement *C9*

In chapter 4 we go into detail on the functional analyses performed on serum samples of individuals carrying rare variants in *C9*, and HEK293F cells in which the same *C9* mutations were introduced.

Chapter 5: Geographic distribution of rare genetic variants previously associated with age-related macular degeneration

For chapter 5 we describe the geographical distribution of seven independently associated rare variants for ~40.000 individuals.

Chapter 6: Phenotype characteristics of patients with age-related macular degeneration carrying a rare variant in the *CFH* gene

Chapter 6 assesses the distinctive phenotypical characteristics of AMD patients carrying a rare genetic variant in *CFH* compared to AMD patients who do not carry such rare variants.

Chapter 7: Genetic screening for macular dystrophies in patients clinically diagnosed with dry age-related macular degeneration

In chapter 7 we evaluate the occurrence of AMD-mimicking dystrophies in an AMD cohort. We screened individuals with intermediate and advanced geographic AMD for genes associated with autosomal dominant and autosomal recessive macular degeneration mimicking AMD.

Chapter 8: Genotype-phenotype correlations of low frequency genetic variants in the complement system in renal disease and age-related macular degeneration

Genetic variation in the complement system have been linked to renal diseases atypical hemolytic uremic syndrome and C3 glomerulopathy. In chapter 8, we systematically compare low frequency genetic variants found in *CFH*, *CFI*, and *C3* for renal diseases patients, AMD patients and control individuals. In addition, we provide a comprehensive genotype-phenotype correlation analyses between these disease groups

Chapter 9: Whole-exome sequencing in age-related macular degeneration identifies rare protein-altering variants in *COL8A1*, a component of Bruch's membrane

In chapter 9 we used whole-exome sequencing to detect a disease burden of rare protein-altering variants in a large European cohort consisting of 1125 AMD cases and 1361 controls individuals.

Chapter 10: General discussion

The discussion paragraph elaborates on the primary findings of this thesis, places these findings in a broader perspective, and discusses the clinical and scientific relevance.

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Maartje J. Geerlings
Eiko K .de Jong
Anneke I. den Hollander

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THE COMPLEMENT SYSTEM IN AGE-RELATED MACULAR DEGENERATION: A REVIEW OF RARE GENETIC VARIANTS AND IMPLICATIONS FOR PERSONALIZED TREATMENT

CLINICAL CHARACTERISTICS OF AGE-RELATED MACULAR DEGENERATION

Age-related macular degeneration (AMD) is the leading cause of irreversible vision loss among the elderly, accounting for 8.7% of blindness worldwide. AMD is most prevalent in populations of European ancestry with approximately 1-3% of the total population suffering from an advanced form of AMD.[1-3] Globally, the total number of patients with any type of AMD is expected to increase over the next 25 years to 288 million affected individuals.[3]

The disease is characterized by a gradual loss of central vision due to photoreceptor cell degeneration in the centre of the retina at the back of the eye, known as the macula. Photoreceptors are in close contact with a layer of cells called the retinal pigment epithelium (RPE). RPE cells support the function of the photoreceptors and play an important role in maintaining retinal homeostasis. In AMD, this natural function of the RPE is disturbed, resulting in the accumulation of retinal waste products called drusen underneath the RPE. Drusen are the tell tale sign of AMD and are easily recognized by ophthalmologists.

AMD is a progressive retinal disease is which the early stage is characterized by relatively few small drusen within the macula. When AMD progresses, drusen size and number increase, eventually leading towards more advanced stages of AMD. Two forms of advanced AMD are distinguished. The first form, neovascular AMD, is characterized by infiltration of abnormal blood vessels into the retina. These newly formed vessels are fragile and when they break, the leakage of blood constituents in the retina leads to sudden vision loss. The second form of advanced AMD, geographic atrophy, is the result of gradual degeneration of the RPE and photoreceptors cells. Although neovascularization occurs in only 15-20% of AMD cases, it is responsible for the vast majority of vision loss caused by AMD. Drugs targeting vascular endothelial growth factor (VEGF), one of the central molecules in neovascularization, have proven to be very successful in neovascular AMD. However, no treatment is available for the remaining majority of early, intermediate or geographic atrophy AMD cases, and furthermore there are no effective means of preventing progression of early to advanced stages.[4,5]

THE COMPLEMENT SYSTEM PLAYS A CENTRAL ROLE IN THE ETIOLOGY OF AMD

Research on the etiology of AMD: a historical perspective

Today it is known that AMD is the result of a complex interaction of environmental and genetic risk factors. Pooled evidence from numerous studies has demonstrated that environmental factors like aging itself, smoking behavior, and body mass index (BMI) are strong risk factors

for AMD. In addition, cataract surgery, cardiovascular disease and family history are also strongly associated.[1] Before any specific gene or biological pathway had been conclusively linked to AMD, studies into the molecular constituents of drusen had already suggested that AMD may have an immunological component. This suggestion arose after proteins involved in inflammation and/or other immune-associated responses, including components of the complement system, were found within drusen.[6-8]

Evidence for a strong genetic component in AMD arose from twin and family studies. Twin studies observed a high concordance of AMD between monozygotic pairs, even double compared to dizygotic pairs, and estimated that the heritability of AMD may be as high as 45 to 70%.[9-11] These findings were in line with familial aggregation analyses that observed a higher prevalence of AMD characteristics and an earlier onset of disease symptoms among relatives of patients compared to control families.[12,13]

Genetic evidence for a role of the complement system in AMD

In search for genomic regions implicated in AMD, genetic linkage analyses were done in large family-based studies.[14-18] Among a few other regions, the findings from these studies strongly and consistently implicated a region on chromosome 1 in the disease. When the first genome-wide association study (GWAS) for AMD was performed in 2005, it identified that same genomic region, which lead to the discovery of a highly associated genetic variant in complement factor H (*CFH*; p.Tyr402His).[19] These findings were corroborated by three additional studies.[20-22]

Table 1: Genes in the complement system associated with AMD

Gene/Locus	Approach	Reference#
C2/CFB	Candidate gene	[23]
C3	Candidate gene/WGS	[24,25]/[26-28]
C9	Candidate gene	[27,29]
CFH	Candidate gene/ Linkage/ GWAS	[19-22]/[30]
CFHR1-CFHR3	Candidate gene	[31]
CFI	Candidate gene	[32]/[33]
VTN	GWAS	[34]

# Reference of first cited association based on common and/or rare genetic variant.
WGS = whole-genome sequencing; GWAS = genome-wide association study

Through genetic studies that followed over the next decade, the understanding of the genetic basis of AMD increased dramatically with the identification of disease-associated variants across several biological systems.[35] The genetic link between AMD and the complement system

was further expanded when genetic variants in or near complement factor I (*CFI*), complement component 3 (*C3*), complement component 2 (*C2*), complement component 9 (*C9*), complement factor B (*CFB*) and vitronectin (*VTN*) were also found to be associated with the disease.[23-25,32,34,35] (**Table 1**). In addition, a common haplotype carrying a deletion of complement factor H related genes *CFHR1* and *CFHR3* was found to be protective for AMD.[31]

The role of rare genetic variants in AMD

Common genetic variants (with a minor allele frequency (MAF) of >5% in the population) near the complement genes *CFH*, *C2/CFB*, *C3* and *CFI* together explain 40-60% of the heritability of AMD.[36] However, a large fraction of the heritability still remains unknown and is referred to as *missing heritability*. One hypothesis states that low frequency and rare genetic variants (with a MAF of <1-5% and <1%, respectively) may explain the remaining fraction of the heritability.[37] During the past years, genetic studies in AMD have therefore shifted towards the identification of rare genetic variants. However, a practical problem arises when analyzing rare variants. The number of patients and controls needed for the identification of novel variants increases when variants are more rare, since the sample size requirements increase roughly linearly with the inverse of the allele frequency. Therefore, analyses of very large cohorts are required for a comprehensive understanding of the role of rare genetic variants in AMD.

Genetic approaches to identify rare genetic variants in AMD

In order to discover rare variants investigators resort to other methods of analyses than those methods yielding insight into common variation. An effective approach that can be used to detect rare disease-associated variants is through a GWAS using exome chips. An exome chip is an array containing both common genetic variants as well as rare exonic variants, and is cost-effective in capturing a specific set of variants in large case-control studies. These chips can be customized and enriched for specific variants of interest. The approach is limited in the sense that it cannot discover new genetic variants other than the ones that the chip captures, but after imputation the chip covers over 12 million variants across the genome.[34] A recent large GWAS using exome chips detected 52 (45 common and 7 rare) variants at 34 genomic regions that are independently associated with AMD. More than one third (19/52) of these variants reside in or near a gene of the complement system: *C2/CFB, C3, C9, CFH, CFI,* and *VTN* (**table 1**). Besides evaluating the association of single genetic variants with the disease, the cumulative number of rare variants detected across an entire gene can be compared between patients and control individuals using burden tests. Interestingly, a significant burden of rare variants in the *CFH* and *CFI* genes, in addition to two other genes (*TIMP3* and *SLC16A8),* was observed in AMD.[34]

Another approach that is widely used to detect rare variants is sequence analysis of candidate genes in cases and controls. An advantage of this approach above the use of exome chips is that it can discover new genetic variants, thereby allowing a comprehensive analysis of all

genetic variation in a candidate gene or a set of candidate genes. With the development of next-generation sequencing technologies, tens to hundreds of genes can effectively be analyzed in large cohorts consisting of thousands of individuals. The candidate gene approach has been successfully employed in AMD in several studies, which have mainly focused on sequencing of genes of the complement system and other genes previously associated with AMD. These studies lead to the discovery of rare variants in the *CFH*, *CFI*, *C3* and *C9* genes in AMD.[27,28,33,38,39]

Whereas candidate gene sequencing is a very targeted approach, whole exome sequencing (WES) or whole genome sequencing (WGS) can interrogate genetic variants in all coding regions of the genome (WES) or even the entire genome (WGS). Since WES and WGS are expensive to perform in large cohorts, approaches can be used to enrich for rare variants, for example by analyzing large AMD families. Recent studies in AMD using WES and WGS have successfully identified novel genetic variants in AMD using a case-control cohort[26] or by analyzing multiple affected individuals of large AMD families.[40-46] New genetic variants were detected in *CFH*, *CFI*, *C3* and *C9*, in addition to other genes (*FBN2* and *HMCN1*). Although rare variants segregated with AMD in some of these families,[41,43,44] several variants did not perfectly segregate with the disease, but were enriched in cases compared to control individuals.[40,42,45] This is in line with the complex etiology of AMD, to which both common and rare genetic variants, and also environmental factors contribute.

RARE GENETIC VARIANTS IN THE COMPLEMENT SYSTEM

Multiple rare genetic variants in the complement system have been associated with AMD. The following paragraphs summarize these variants, focusing on the ones that were found in more than a single AMD patient. A complete list of rare variants described in literature is presented in **Supplementary Table 1** and visualized in **Figure 1**.

Complement factor H

An important rare variant associated with AMD, *CFH* p.Arg1210Cys, was discovered after re-sequencing a rare risk haplotype in *CFH*.[30] The authors demonstrated that the p.Arg1210Cys variant was highly associated with AMD, independently of the common variant p.Tyr402His. Moreover, carriers of this variant were significantly younger when the first symptoms of AMD appeared. The p.Arg1210Cys variant conferred a 47 times higher risk of developing AMD.[34]

Earlier, nonsense variant p.Gln408Ter and missense variant p.Arg1078Ser in *CFH* had already been identified in families that presented with a particular subtype of AMD known as cuticular drusen. Here it was argued that the rare variant, in addition to the common variant *CFH* p.Tyr402His, may underlie the phenotype.[47] In addition, in other families with cuticular drusen, frameshift variants p.Ile184Leufs*33 and p.Lys204Thrfs*26 were identified, also independently of *CFH* p.Tyr402His.[48]

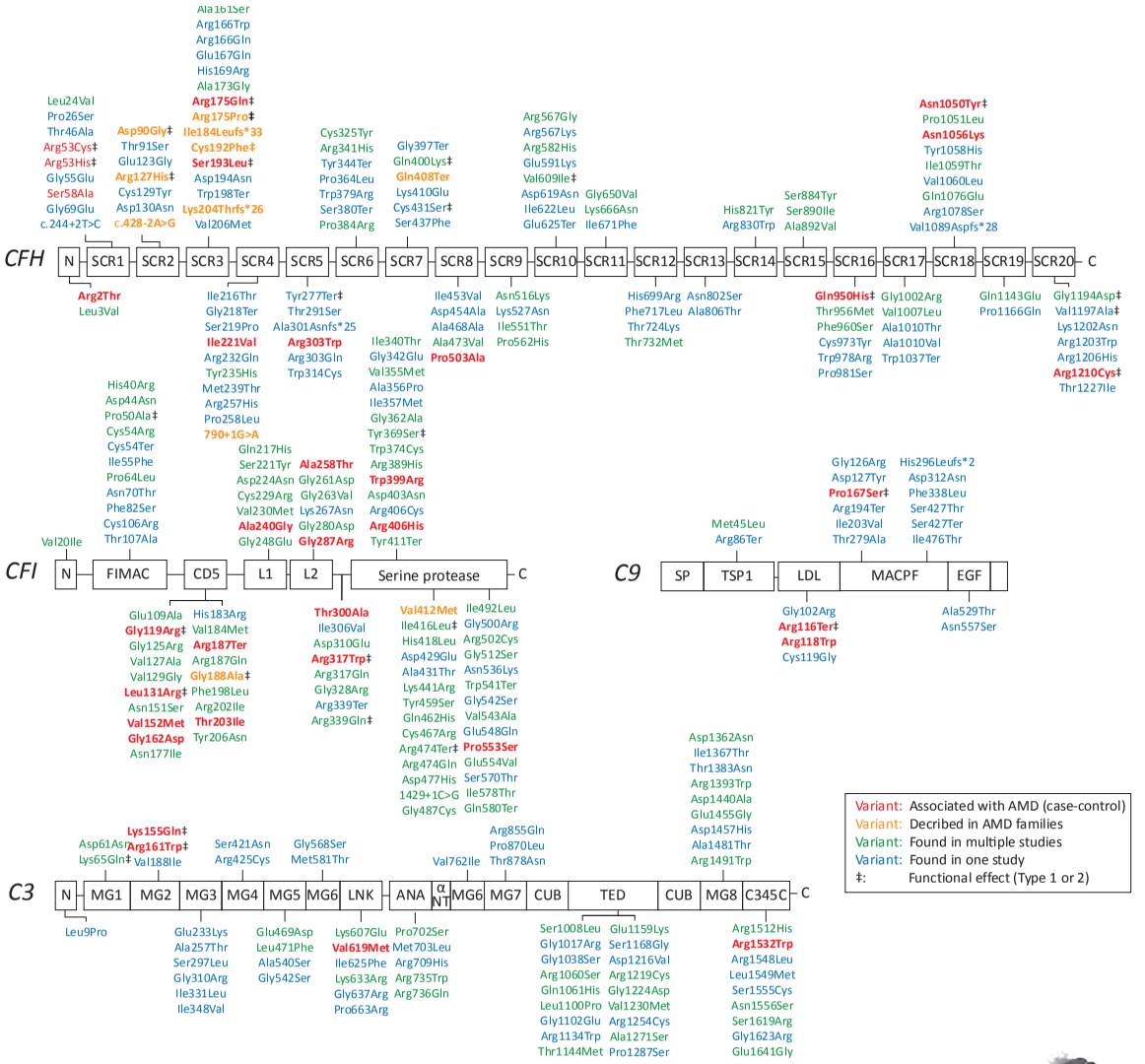

Figure 1: Rare coding variants in *CFH*, *CFI*, *C3* and *C9* found in AMD patients. Variants are color-coded as follows: notated significantly associated with AMD in one or more AMD case-control cohorts (in red), were found in AMD families (in orange), were found in more than one AMD cohort (in green), or were found in one AMD cohort in blue. Variants notated with ‡ have a functional effect on the protein or change systemic levels. CFH: Complement Factor H; *CFI* Complement Factor I; *C3* Complement *C3*; *C9* Complement *C9*.

Splice site variant *CFH* c.790+1G>A and coding variants *CFH* p.Arg53Cys, p.Asp90Gly, p.Arg127His, p.Arg175Pro, p.Arg175Gln, p.Cys192Phe, and p.Ser193Leu were identified by WES of AMD families in which known genetic risk factors could not explain the high burden of disease.[43,45,49] Variant *CFH* p.Pro503Ala was identified using WES in an Amish family after exclusion of the other main risk variants for AMD, and was significantly associated with AMD in an Amish AMD case-control cohort (**Table 2**).[40] Next-generation sequencing was performed for *CFH* in a cohort of 2417 individuals, demonstrating an enrichment of rare variants in functional domains of factor H in AMD. In this study, 65 coding *CFH* variants were identified of which only 15 rare variants were found in more than one affected individual; the other variants were found only in single cases (**Table 3, Supplementary Table 1**).[38]

Overall, three different splice site variants, ten different nonsense, four different frameshift and 106 different rare missense variants in *CFH* were detected in AMD case-control and family studies (**Supplementary Table 1**). Functional variants seem to cluster in SCR 1-4 domains which mediate complement regulation of the protein, and SCR 19-20 which allow attachment of FH to the host cell. In total, 10 of 15 identified functional variants affect amino acid residues in one of these domains. In total, 124 variants were found of which 14 were significantly ($p < 0.05$) associated with AMD (**Table 2**). The majority of the coding variants (68/124) were found in only in one study cohort and were not significantly associated with AMD.

Complement factor I

After sequencing the entire *CFI* gene in a subset of patients, and subsequent replication in a number of large case-control cohorts, the variant p.Gly119Arg was shown to be strongly associated with AMD.[33] The p.Gly119Arg variant conferred a 5 times higher risk of developing AMD.[34]

Additional variants in *CFI* have been identified in AMD families, including p.Gly188Ala,[33] p.Leu131Arg,[45] and p.Val412Met.[44] It has been demonstrated that the *CFI* gene is enriched for rare variants four-fold in AMD cases compared to controls, and that these variants largely reside in the catalytic domain (residing in the serine protease domain) of the protein.[27] Of the 70 variants identified in this study,[39] eight coding variants were confirmed in five or more individuals, while the majority of variants were found only once (**Supplementary Table 1**). None of the variants were individually associated with AMD, although three variants showed a nominal association (*CFI* p.Pro553Ser, p.Arg406His and p.Ala240Gly)(**Table 2**).

Overall, one splice site variant, seven different nonsense, and 86 different rare missense variants in *CFI* were detected in AMD case-control and family studies (**Supplementary Table 1**). The variants appear to cluster in the serine protease domain, with 42 of 94 identified variants affecting amino acid residues in this domain, which is in accordance with a previous report.[27] Of these variants, 14 were significantly associated with AMD (**Table 2**).

Table 2: Rare variants in complement genes associated with AMD and accompanying OR/LOD scores.

Gene	Variant (p.)	Effect (Odds Ratio)	Significance (P-value and/or LOD score)	Study
CFH	Arg2Thr	Risk (14.1)	P = 0.0158**	34
CFH	Arg53Cys	Risk (22.5)	LOD score 5.07, P = $6.7x10^{-7}$, P = 0.00118**	34,43
CFH	Arg53His	Risk (13.4)	P = 0.01**	34
CFH	Ser58Ala	Risk (2.58)	P = 0.00702**	34
CFH	Asp90Gly	Risk (NA)	LOD score 1.22, P = 0.009	43
CFH	Arg175Gln	Risk (1.50)	P = 0.04	51
CFH	Ser193Leu	Risk (NA)	P = 0.01	51
CFH	Ile221Val	Risk (11.8)	P = 0.0314**	34
CFH	Arg303Trp	Risk (12.2)	P = 0.0378**	34
CFH	Pro503Ala	Risk (NA)	P = 9.27×10^{-13}	40
CFH	Gln950His	Protective (0.72)	P = 0.00258**	34
CFH	Asn1050Tyr	Protective (0.36)	P = $5.92x10^{-44}$**	34
CFH	Asn1056Lys	Protective (0.08)	P = 0.024**	34
CFH	Arg1210Cys	Risk (31.8)	P = $3.2x10^{-31}$*	34
CFI	Gly119Arg	Risk (3.87)	P = $8.6x10^{-11}$*	34
CFI	Leu131Arg	Risk (NA)	P = 0.02	51
CFI	Val152Met	Risk (7.57)	P = $4.65x10^{-4}$**	34
CFI	Gly162Asp	Risk (20.3)	P = 0.00231**	34
CFI	Arg187Ter	Risk (1.30)	P = 0.0175**	34
CFI	Thr203Ile	Risk (2.46)	P = 0.0344**	34
CFI	Ala240Gly	Risk (OR 7.43)	P = 0.02	39
CFI	Ala258Thr	Risk (3.88)	P = $6.25x10^{-5}$**	34
CFI	Gly287Arg	Risk (4.61)	P = 0.00761**	34
CFI	Thr300Ala	Protective (NA)	P = 0.0144**	34
CFI	Arg317Trp	Risk (12.2)	P = $1.97x10^{-4}$**	34
CFI	Arg339Gln	Risk (11.8)	P = 0.0312**	34
CFI	Arg406His	Protective (0.10)	P = 0.02	39
CFI	Val412Met	Risk (NA)	LOD score 2.51	44
CFI	Pro553Ser	Risk (3.7; 2.69)	P = 0.04; P = 0.03	39,51
C3	Lys155Gln	Risk (3.12)	P = $1.5x10^{-32}$*	34
C3	Arg161Trp	Risk (1.5)	P = 0.01	51
C3	Val619Met	Risk (2.66)	P = $2.38x10^{-4}$**	34
C3	Arg1532Trp	Risk (12.3)	P = 0.0379**	34
C9	Arg116Ter	Protective (0.20)	P = 0.021	29
C9	Arg118Trp	Risk (1.12)	OR = 0.04	51
C9	Pro167Ser	Risk (1.79)	P = $1.6x10^{-14}$*	34

*IAMDGC Locus-wide conditioned analysis (adjusting for the identified index variant(s) in the locus) ** Not conditioned for index variant(s) in the locus. NA = not available.

Complement component 3

The p.Lys155Gln variant in *C3* was described to be associated with AMD independently by three studies.[26-28] The p.Lys155Gln variant confers a 3 times higher risk of developing AMD.[34]

In a sequencing study of all coding exons of the *C3* gene, four other C3 variants were identified: p.Lys65Gln, p.Arg161Trp, p.Arg735Trp and p.Ser1619Arg.[50] All but p.Arg161Trp were found to be associated in the index cohort, but none of the rare variant associations were replicated in an independent cohort. The p.Arg735Trp and p.Ser1619Arg variants and two additional variants, p.Val619Met and p.Lys633Arg, were identified by next-generation sequencing of the *C3* gene in 1676 cases and 745 controls, but none of the variants were found to be significantly associated with AMD.[27]

Overall, 71 different rare missense variants in *C3* were detected in AMD case-control and family studies (**Supplementary Table 1**). The variants that effect protein function are located at the first and second MG domains. Of these variants, four variants were significantly associated with AMD (**Table 2**). The majority (39/71) of variants were found in only one in one study cohort and were not significantly associated with AMD.

Complement component 9

Sequence analysis of the *C9* gene in 1676 cases and 745 controls demonstrated that the p.Pro167Ser variant confers an increased risk of developing AMD.[27] The p.Pro167Ser variant was confirmed to confer risk of AMD in other cohorts as well,[42] with a 1.7 times increased risk of developing AMD.[34]

Sequence analysis of *C9* also identified 2 other variants, p.Met45Leu and p.Ile203Val, but these were not found to be significantly associated with AMD.[27] In addition, variant p.Arg118Trp was identified in an AMD family with three affected siblings.[45] The nonsense variant p.Arg95Ter has been associated with a reduced risk for advanced AMD but is a founder mutation of East Asian origin and extremely rare in European populations.[29]

Overall, four nonsense, one frameshift and 15 different rare missense variants in *C9* were detected in AMD case-control and family studies (**Supplementary Table 1**). Of these variants, three were significantly associated with AMD (**Table 2**). The majority (16/20) of variants were found in one study cohort and were not significantly associated with AMD.

FUNCTIONAL IMPLICATION OF RARE GENETIC VARIANTS

A genetic association provides statistical evidence that a particular variant is implicated in the disease, but if offers no insight into the molecular mechanisms that underlie and lead to the disease. To better understand this, the functional consequences of genetic variants on the complement system need to be investigated. Although the complement system acts locally, complement components or activation products can be detected systemically, for example in serum or plasma. In literature, several studies have described the expression of complement regulators and measurements of both complement components and activation products in AMD patients compared to controls. In addition, for some variants *in vitro* studies have been performed to examine their effect. In the next paragraphs we summarize the rare variants described in AMD literature, and detail their functional effects (**Table 3**).

Complement factor H

Complement factor H (FH) is an inhibitor and plays a key role in the alternative pathway of the complement system. FH protects tissues by inhibiting the formation of excess C3 convertase through competition with factor B (FB) in the binding of C3b, and in addition promoting the decay of surplus C3 convertase.

The p.Arg1210Cys variant showed reduced binding to C3b, C3d and heparin but normal cofactor activity to factor I (FI).[52,53] Through the introduction of a cysteine residue, p.Arg1210Cys forms covalent interactions with human serum albumin.[52] It has been postulated that it is the albumin bound to FH rather than any functional defect of the protein itself that eventually disrupts FH function.[54] Variants p.Arg53Cys and p.Asp90Gly are both located within the first four domains of FH which are known to bind C3b, however only p.Arg53Cys showed minor decreased affinity to bind C3b.[43] Independent of the C3b affinity, the variants strongly affected co-factor activity of FI. In addition, p.Arg53Cys disrupted decay accelerating activity and was shown to correlate to low C3 levels.[43,55,56] Later, variants c.790+1G>A, p.Arg127His, p.Arg175Pro and p.Cys192Phe were analyzed for levels of serum concentration, and all variants, except p.Arg127His, had reduced FH serum levels compared to a control group. The coding variants all shown impaired protein secretion.[49,57]

Variants can be grouped according to effect on the protein function. Type 1 mutations cause lower protein expression levels as a result of misfolding or degradation of the protein, in contrast to type 2 mutations that result in reduced functionality which is not necessarily reflected in protein levels. This distinction is also observed for variants found in *CFH*. Serum concentration of FH and C3 were measured in plasma samples of carriers of 5 *CFH* variants (p.Cys192Phe, p.Tyr277Ter, p.Cys431Ser, and two splice-site variants). For these variants, lower FH concentrations were observed in each of the carriers compared to a non-carrier

control set,[38] and can thus be classified as type 1 mutations. For two other variants, p.Arg175Gln and p.Ser193Leu, serum levels were normal but these variants exhibited a reduced ability to degrade C3b,[45] suggesting that they are type 2 mutations.

Complement factor I

Complement factor I (FI) is a serum serine protease that converts C3b and C4b to their inactive form to reduce the formation of the C3 and C5 convertases. Unbound C3b would otherwise result in increased C3 convertase formation and feedback amplification of the alternative pathway.

Overall, many rare variants in *CFI* result in lower FI levels in serum and consequently lower the regulatory activity of the alternative pathway.[27,39] Serum measurements found low FI levels for pathogenic variants p.Ala240Gly and p.Gly119Arg compared to non-carriers, while serum levels were normal for p.Pro553Ser and p.Arg406His.[39]

In an independent study, *CFI* p.Gly119Arg and p.Gly188Ala resulted in reduced FI levels in plasma, which was supported by *in vitro* analysis of recombinant FI in human cells, showing that mutant FI is expressed and secreted at lower levels than wild-type FI.[33] Overall plasma samples of carriers of the p.Gly119Arg variant showed a lower ability to degrade C3b compared to non-carriers, but the ability of recombinant p.Gly119Arg mutant protein to cleave C3b and C4b was intact. This suggests that this variant is a type 1 mutation and that low expression levels underlie the observed functional effect.[33] Similarly, variant p.Leu131Arg in *CFI* showed both impaired FI levels and an inability to properly cleave C3b,[45] supporting that is also a type 1 mutation. A difficult variant to classify is *CFI* p.Pro553Ser, which confers risk for AMD in multiple studies.[39,45] The variant however does not alter system FI levels, is classified by prediction software as benign, and showed a lower ability to degrade C3b compared to non-carriers controls but not non-carriers cases.

Table 3: Described functional effects of rare variants in the complement system

Gene	Variant (p.)	Functional implication	Sources
CFH	**Arg53Cys**	Reported to possibly affect the local conformation of FH. This variant did not affect levels of FH in serum of 22 individuals, but showed slightly reduced binding affinity to C3b compared to wild type. Marked loss of decay accelerating activity. Trend towards lower cofactor activity for FI.	[43]
	Arg53His	Like the 53Cys variant, the 53His variant showed minor decreased affinity to bind C3b. Independent of the C3b affinity, the variant strongly affected co-factor activity of FI. In addition, the variant disrupted decay accelerating activity and was shown to correlate to low C3 levels.	[58,59]
	Asp90Gly	No reported effect on FH levels in serum from 22 individuals. No effect on C3b binding affinity and decay accelerating activity. Significantly reduced cofactor activity for FI	[43]
	Arg127His	Reduced FH serum levels in heterozygous and homozygous carriers and no secretion of the protein.	[49,57,60]
	Arg175Pro	Reduced FH serum levels and no secretion of the recombinant protein.	[49]
	Arg175Gln	No reported effect on FH levels. Reduced C3b degradation ability.	[45]
	Cys192Phe	Lower expression of FH and reduced secretion of the protein. Normal C3 in plasma of one carrier	[38,49]
	Ser193Leu	No reported effect on FH levels. Reduced C3b degradation ability.	[45]
	Tyr277Ter	Lower expression of FH and normal C3 in plasma of one carrier	[38]
	Arg303Gln	Normal plasma levels for FH, FI and C3	[61]
	Gln400Lys	Lower FH levels, but no effect on plasma concentrations of C3 and FB	[62]
	Cys431Ser	Lower expression of FH and normal C3 in plasma of one carrier	[38]
	Val609Asp	Affects FH expression and resulted in decreased alternative pathway activity and C3 level in remission	[63]
	Ser890Ile	The variant did not result is differences in FH co-activity with FI. The C3b binding was not affected and FH concentration in plasma were normal. In addition a hemolytic assay showed that the capacity to regulate the alternative pathway on cellular surfaces was normal.	[64]
	Gln950His	This variant demonstrated reduced erythrocyte binding and, consequently, increased lysis after serum addition to sheep erythrocytes. Patient plasma levels of FH were not different compared to controls, but transient expression levels of mutant lagged behind that of the wild type. No impaired cofactor binding for FI was observed and normal complementary inhibitory functions were observed.	[63,65]
	Thr956Met	No effect on C3 or FH levels in plasma. The lysis of erythrocytes was not increased and no effect on protein expression was shown.	[63,66]
	Val1007Leu	No differences in FH co-activity nor C3b binding; normal hemolytic assay (capacity to regulate the alternative pathway on cellular surfaces); normal FH in plasma	[64]

	Asn1050Tyr	Abnormal C3 and normal FH levels in serum	[67]
	Gln1076Glu	Normal C3 and FH levels in serum	[68]
	Gly1194Asp	Slightly increased complement regulatory function of mutant FH on cell surfaces (sheep erythrocyte lysis); normal C3 FH and FI levels in serum	[69,70]
	Val1197Ala	Normal lysis of sheep erythrocytes, low FH and C3 levels, and shows low binding to surface bound C3b.	[52,71]
	Arg1203Trp	Hemolytic test showed no lysis	[63]
	Arg1210Cys	This variant results in a covalent binding to human serum albumin which hampers all FH functional domains. It also shows reduced binding to heparin and endothelial cells and binding to C3b and C3d is also decreased. No effects on cofactor activity for FI was reported and no effect on erythrocyte lysis was shown.	[52-54,72,73]
	c.244+2T>C splice site	Normal expression of FH and high C3 in plasma of one carrier	[38]
	c.790+1G>A; splice site	Lower expression of FH in three carriers and low C3 in plasma of one carrier	[38,49]
CFI	Pro50Ala (Pro32Ala)*	Elevated FB in plasma; normal C3 and FI in plasma; impaired function towards degradation of the alpha-chains of C4b and C3b in solution when FH was used as cofactor	[74]
	Gly119Arg (Gly101Arg)*	This variant resulted in reduced FI levels in human serum as well as in transient in vitro expression studies. The variant resulted in a lower ability to degrade C3b due to impaired expression and secretion of the mutant protein.	[33,39,45]
	Leu131Arg (Gly113Arg)	The variant resulted in a lower ability to degrade C3b which could be due to impaired expression and secretion of the mutant protein.	[45]
	Gly188Ala (Gly170Ala)*	This variant resulted in lower FI levels in human serum as well as in transient in vitro expression studies. The variant resulted in impaired degradation of C3b.	[33]
	Arg202Ile (Arg184Ile)*	This variant had no effect on FI levels in human serum	[39]
	Ala240Gly (Ala222Gly)*	This variant resulted in lower or normal FI levels in human serum/plasma. The degradation of fluid phase C4b and C3b was normal, although the ability to cleave surface-bound C3b was impaired.	[39,75,76]
	Gly261Asp (Gly243Asp)*	No effect on FI levels in human serum; slightly different migration pattern; normal degradation of C3b and C4b.	[39,77,78]
	Thr300Ala (Thr282Ala)*	No effect on FI levels in human serum	[39]
	Arg317Trp (Arg299Trp)*	Normal FI plasma level and normal functioning on hemolytic assay; only impaired secretion compared to wildtype FI.	[75,76,78]
	Arg339Gln (Arg321Gln)*	Reduced C3, FH, and FB levels, but normal FI levels in serum	[63]
	Ile340Thr (Ile323Thr)*	Normal FI and C3 levels in serum	[70]
	Tyr369Ser (Ile351Thr)*	Normal FH and C4 levels; low C3 levels in serum	[79]
	Arg406His (Arg388His)*	No effect on FI levels in human serum	[39]

	Ile416Leu (Ile398Leu)*	Low FI and C3 serum levels; normal FB levels	74,80
	His418Leu (His400Leu)*	Homozygous variation results in FI deficiency (low or undetectable FI and C3 levels)	81
	Lys441Arg (Lys423Arg)*	This variant had no effect on FI levels in human serum	39,82
	Tyr459Ser (Tyr441Ser)*	Normal FI and C3 levels in serum	74
	Arg474Gln (Arg456Gln)*	Normal FI protein level.	63
	Arg474Ter (Arg456Ter)*	Low FI and C3 serum levels; normal FB levels	74,76,83
	Pro553Ser (Pro535Ser)*	This variant had no effect on FI levels in human serum and slightly lower ability to degrade C3b.	39,45
C3	**Lys65Gln (Lys43Gln)***	This variants weakened the interaction of C3b and FH and showed reduced MCP binding affinity	84,85
	Lys155Gln (Lys131Gln)*	This variant resulted in significantly reduced cleavage of C3b in fluid phase cofactor assays as well as reduced binding to FH. MCP cofactor activity was not changed.	26-28,34
	Arg161Trp (Arg139Trp)*	Reduced binding activity of C3b to FH in one study and no effect on binding and cleavage of C3 in other studies. MCP binding was reduced, FB binding was increased. This variant is discussed to be a gain-of-function variant of the convertase complex and C3a, C5a, C5b-9 formation was shown to be increased.	26-28,34
	Arg735Trp (Arg713Trp)*	This variant showed no functional effects on MCP binding, FI cofactor activity, FB binding, CR1 binding and FH binding.	88,89
	Leu1549Met (Leu1527Met)*	No influence on FH, MCP, or CR1 binding	85
C9	**Arg95Ter**	C9 serum concentration was below the level of detection	90,91
	Pro167Ser	Median C9 serum concentration was elevated in carriers compared to non-carriers	45

Only variants on which functional analysis were done are shown * described in literature without signaling peptide Bold: genetic association with AMD through case-control analysis or found in multiple cases of within an AMD family

Complement component 3

Complement factor 3 (C3) is the central player in the activation of the complement system and several rare variants in *C3* have been investigated functionally (**Table 3**).

The C3 p.Lys155Gln variant is located close to the binding site for FH, and its interaction was analyzed both *in silico* and *in vitro* with matching results. The p.Lys155Gln variant causes inefficient binding of C3 with FH and consequently reduces cofactor mediated cleavage of C3b.[27,92] Variant p.Lys65Gln leads to a decreased binding of FH to C3b and a slightly lowered affinity to the membrane cofactor protein (MCP; also known as CD46). The p.Arg161Trp

variant increases the affinity to bind to FB and thereby creates an overactive C3 convertase accompanied by increased formation of C3a, C5a and C5b-C9. In addition, p.Arg161Trp also has reduced binding affinities for MCP and FH, which would otherwise both inactivate C3b/C4b through co-factor activity with FI.[84-86] Variant p.Arg735Trp demonstrated normal MCP, FB, sCR1 and FH binding and proper cleavage by FI.[88]

Complement component 9

Complement factor 9 (C9) takes part in the formation of the terminal complement complex (TCC) comprised of several C5b-9 elements. The TCC can be soluble or it can form a scaffold on the surface of the membrane together with multiple (up to 16) C9 proteins to assemble a pore-like structure known as the membrane attack complex (MAC) promoting cell lysis.

Recently it was shown that carriers of the C9 p.Pro167Ser variant, associated with an increased risk for AMD, have elevated C9 concentrations in serum compared to non-carriers. It was hypothesized that increased C9 levels could result in elevated complement activation which, through lysis of the cells, may contribute to the degenerative process observed in AMD.[45] Asian Founder mutation *C9* p.Arg95Ter is responsible for most Japanese C9 deficiency cases but is simultaneously protective for AMD. It is shown that C9 serum concentrations are low, even below detection level,[90,91] suggesting that less MAC can be formed which could otherwise contribute to retinal damage.

COMPLEMENT THERAPIES IN AMD

The treatment of neovascular AMD has highly improved with the introduction of anti-neovascularization therapy with VEGF as the principle target. However, VEGF-based treatment is not effective or even not applicable in most AMD patients since only a minority of patients suffer from the neovascular form of AMD. Currently, no available treatment is available for the majority of AMD patients that suffer from early or intermediate AMD or geographic atrophy. Moreover, no effective means other than a modest effect of AREDS supplements to reduce the risk of AMD progression is available.[93-95] Because of the central role of the complement system in AMD, complement inhibition has been considered a potential therapeutic option and several clinical trials have been initiated to investigate this possibility (**Table 4**).[96-98]

Table 4: Clinical trials for AMD targeting the complement system

Drug, trade name (company)	Target	Status	Source
Eculizumab, Soliris (Alexion)	Complement component 5	Phase II has been completed	[97]
Lampalizumab (Genentech, Roche)	Complement factor D	Phase II has been completed; recruitment for phase III clinical trial has started	[96,98]
Avacincaptad pegol (ARC-1905) Zimura (Ophthotech)	Complement component 5	Recruitment for II/III clinical trial has started.	Clinicaltrials.gov
Tesidolumab , LFG316 (Novartis)	Complement component 5	Recruitment for II/III clinical trial has started	Clinicaltrials.gov
CLG561 (Novartis)	Properdin	Recruitment for phase II clinical trial has started	Clinicaltrials.gov
POT4, (Potentia Pharmaceuticals and Alcon)	Complement component 3	Phase I has been completed.	Clinicaltrials.gov

Of the studies that published the outcomes, the COMPLETE study was a phase II clinical trial with systemic eculizumab, an humanized IgG antibody that inhibits complement component 5 (C5). The trial results showed that eculizumab was not effective in the treatment of geographic AMD, as the growth of atrophic lesions did not decrease after 6 months of treatment.[97] The MAHALO study was a phase II clinical trial with lampalizumab, an antibody directed against complement factor D. MAHALO showed promising preliminary results: progression of the geographic atrophy lesion showed a 20% reduction after 18 months of treatment and it was suggested that lampalizumab is most effective in a subpopulation of patients, since an even higher reduction rate was seen in patients carrying a specific *CFI* genotype.[96,98] A phase III trial with lampalizumab is currently ongoing, which will further investigate the role of the *CFI* genotype on treatment response.

In summary, a number of clinical trials using complement inhibitors in AMD have been performed, or are currently still running. Eculizumab seemed not to be effective, while lampalizumab may have a (limited) beneficial effect in reducing AMD progression.

DISCUSSION AND CONCLUSION

Just over a decade after the initial discovery of the involvement of *CFH* in AMD,[19] basic science has been translated to experimental approaches where complement inhibitors against AMD are now tested in clinical trials. Although the involvement of the complement system in AMD has been firmly established, the limited success of these clinical trials seem to suggest that the drugs currently tested are not entirely effective in the overall study populations.[96] In part this may be explained by the fact that, besides the complement system, also other biological systems like the extracellular matrix, lipid homeostasis or oxidative stress may contribute substantially to AMD pathogenesis.[34,94,99] At this time, it is not well understood how these pathways interact with each other in the development of the disease. Therefore the complement system is not necessarily the only, or even an appropriate target for any given AMD patient.

It is conceivable that a subset of patients would benefit more from anti-complement therapy than others, in particular those that have a genetic defect in genes of the complement system. It has now been shown that rare genetic variants in complement genes that are genetically associated with AMD often negatively impact the functioning of this system.[33,38,39,43,45] However, does this mean that all AMD patients carrying a rare variant in the complement system will benefit from complement inhibitors?

Several issues arise when considering the role of rare variants in AMD. To date only few rare variants have been consistently replicated across multiple cohorts.[26-28,30,33,34] While some rare variants are present relatively abundantly in one population, they are virtually absent in other populations, for example p.Arg1210Cys in *CFH*[28,30,100,101] and p.Gly119Arg in *CFI*.[33,39,102,103]

Many other potentially interesting variants have been found, illustrated by this review, but very large cohorts are required to detect a significant genetic association with AMD.[37,104] The question arises whether a rare variant is or is not relevant in the context of the disease if it cannot be genetically associated with AMD. Functional assays could help clarify if a genetic variant has an impact on protein stability or systemic levels. This may provide functional evidence that a variant could be involved in the pathogenesis of the disease in the cases where statistical tests are underpowered to detect any association.

At this time, patients who carry AMD-associated rare variants proven to have a negative impact on function, could be prioritized in clinical trials with complement inhibitors. Across multiple cohorts, numerous of such patients have now been identified. Such focused studies would offer a proof of principle that could later benefit many more patients that have a defective complement system based on functional tests, but carry genetic variants that are too rare to be statistically associated with AMD.

AMD patients should therefore be screened using a functional complement assay in addition to genetic analyses. An exciting future lies ahead in the field of AMD where, for each individual patient, genetic evidence and functional tests come together in a treatment plan that is personalized and tailored to the specific needs and requirements of that patient.

Supplementary Table 1: Coding rare variants found in complement genes of AMD patients

Source	Chr	Position	Gene	cDNA change (c.)	Protein change (p.)	rs-number	Number Cases	Number Controls	MAF Cases (%)	MAF Controls (%)	Families (incl proband)	SIFT	Poly Phen	ExAC (%)	Function	IAMDGC Odds Ratio	IAMDGC P-Value
6	1	196621252	CFH	5G>C	**Arg2Thr**	rs142266551	17814	16129	0.0200	0.0000		D	D	0.0025	None	14.08	0.02
23	1	196621252	CFH	5G>C	**Arg2Thr**	rs142266551	2335	797	0.0214	0.0000		D	D	0.0025	None	14.08	0.02
6	1	196621254	CFH	7C>G	**Leu3Val**	rs139254423	17831	16144	0.0400	0.0400		T	P	0.0241	None	1.06	0.89
4	1	196621254	CFH	7C>G	**Leu3Val**	rs139254423	80	-	0.6250	-		T	P	0.0241	None	1.06	0.89
18	1	196642119	CFH	70C>G	**Leu24Val**	NA	1665	752	0.0000	0.0665		T	B	NA	None	3.16	0.48
23	1	196642119	CFH	70C>G	**Leu24Val**	NA	2335	797	0.0214	0.0000		T	B	NA	None	3.16	0.48
18	1	196642125	CFH	76C>T	Pro26Ser	NA	1665	752	0.0300	0.0000		D	D	NA	None	NA	NA
18	1	196642185	CFH	136A>G	Thr46Ala	rs778461886	1665	752	0.0300	0.0000		D	D	0.0008	None	NA	NA
6	1	196642206	CFH	157C>T	**Arg53Cys**	NA	17832	16144	0.0300	0.0000		D	D	0.0017	Table 3	22.54	1.18E-03
18	1	196642206	CFH	157C>T	**Arg53Cys**	rs757785149	1665	752	0.1201	0.0000		D	D	0.0017	Table 3	22.54	1.18E-03
22	1	196642206	CFH	157C>T	**Arg53Cys**	rs757785149	1676	5045	0.1193	0.0000	1 family with 11 carriers	D	D	0.0017	Table 3	22.54	1.18E-03
23	1	196642206	CFH	157C>T	**Arg53Cys**	rs757785149	2335	797	0.0428	0.0000		D	D	0.0017	Table 3	22.54	1.18E-03
18	1	196642207	CFH	158G>A	**Arg53His**	NA	1665	752	0.0300	0.0000		D	P	NA	Table 3	13.39	0.01
23	1	196642207	CFH	158G>A	**Arg53His**	NA	2335	797	0.0428	0.0000		D	P	NA	Table 3	13.39	0.01
23	1	196642213	CFH	164G>A	Gly55Glu	NA	2335	797	0.0214	0.0000		D	D	NA	None	NA	NA
6	1	196642221	CFH	172T>G	**Ser58Ala**	rs141336681	17828	16142	0.0700	0.0300		T	B	0.0140	None	2.82	7.02E-03
18	1	196642221	CFH	172T>G	**Ser58Ala**	rs141336681	1665	752	0.0601	0.0665		T	B	0.0140	None	2.82	7.02E-03
23	1	196642221	CFH	172T>G	**Ser58Ala**	rs141336681	2335	797	0.0857	0.0000		T	B	0.0140	None	2.82	7.02E-03
14	1	196642255	CFH	206G>A	Gly69Glu	NA	33	27	1.5152	0.0000		D	D	0.0008	None	NA	NA
18	1	196642295	CFH	244+2T>C	Splice site	NA	1665	752	0.0300	0.0000		NA	NA	NA	Table 3	NA	NA
22	1	196643011	CFH	269A>G	Asp90Gly	NA	1676	5045	0.0000	0.0000	1 family with 5 carriers	D	D	NA	Table 3	NA	NA
18	1	196643014	CFH	272C>G	Thr91Ser	rs771527214	1665	752	0.0300	0.0000		T	P	0.0025	None	NA	NA
23	1	196645136	CFH	368A>G	Glu123Gly	NA	2335	797	0.0000	0.0627		T	B	NA	None	0.36	0.53
6	1	196645148	CFH	380G>A	**Arg127His**	rs121913058	17831	16144	0.0100	0.0000		D	D	0.0017	Table 3	5.51	0.23
18	1	196645148	CFH	380G>A	**Arg127His**	rs121913058	1665	752	0.0300	0.0000		D	D	0.0017	Table 3	5.51	0.23
21	1	196645148	CFH	380G>A	**Arg127His**	rs121913058	-	-	-	-	1 family with 2 carriers	D	D	0.0017	Table 3	5.51	0.23

23	1	196645148	CFH	380G>A	**Arg127His**	rs121913058	2335	797	0.0428	0.0000		D	D	0.0017	Table 3	5.51	0.23
18	1	196645154	CFH	386G>A	Cys129Tyr	NA	1665	752	0.0300	0.0000		D	D	NA	None	NA	NA
18	1	196645156	CFH	388G>A	Asp130Asn	rs147002633	1665	752	0.0901	0.0000		T	P	0.0091	None	NA	NA
4	1	196646604	CFH	428-2A>G	Splice site	NA	80	-	0.6250	-		NA	NA	NA	None	NA	NA
6	1	196646654	CFH	476G>A	**Ser159Asn**	NA	17832	16144	0.0200	0.0000		T	P	0.0041	None	4.57	0.09
18	1	196646654	CFH	476G>A	**Ser159Asn**	rs370640334	1665	752	0.0901	0.0000		T	P	0.0041	None	4.57	0.09
4	1	196646659	CFH	481G>T	**Ala161Ser**	rs777300338	80	-	0.6250	-		T	B	0.0041	None	NA	NA
18	1	196646659	CFH	481G>T	**Ala161Ser**	rs777300338	1665	752	0.0300	0.0000		T	B	0.0041	None	NA	NA
18	1	196646674	CFH	496C>T	Arg166Trp	NA	1665	752	0.0601	0.0000		D	B	NA	None	NA	NA
18	1	196646675	CFH	497G>A	Arg166Gln	rs770339409	1665	752	0.0300	0.0000		T	B	0.0280	None	NA	NA
18	1	196646677	CFH	499G>C	Glu167Gln	NA	1665	752	0.0300	0.0000		D	D	NA	None	NA	NA
18	1	196646684	CFH	506A>G	His169Arg	rs768647508	1665	752	0.0000	0.0665		T	B	0.0025	None	NA	NA
4	1	196646696	CFH	518C>G	**Ala173Gly**	NA	80	-	0.6250	-		D	B	NA	None	NA	NA
5	1	196646696	CFH	518C>G	**Ala173Gly**	NA	12	-	4.1667	-		D	B	NA	None	NA	NA
4	1	196646702	CFH	524G>A	**Arg175Gln**	NA	80	-	0.6250	-		T	B	NA	Table 3	NA	NA
7	1	196646702	CFH	524G>A	**Arg175Gln**	NA	1831	1367	0.0546	0.0366	1 family with 8 carriers	T	B	NA	Table 3	NA	NA
18	1	196646702	CFH	524G>C	**Arg175Pro**	rs139360826	1665	752	0.0300	0.0000		T	B	NA	Table 3	NA	NA
21	1	196646702	CFH	524G>C	**Arg175Pro**	rs139360826	-	-	-	-	1 family with 2 carriers	T	B	NA	Table 3	NA	NA
23	1	196646702	CFH	524G>C	**Arg175Pro**	rs139360826	2335	797	0.0214	0.0000		T	B	NA	Table 3	NA	NA
19	1	196646728	CFH	550delA	Ile184 Leufs*33	NA	21	192	2.3810	0.0000	1 family with 6 carriers	NA	NA	NA	None	NA	NA
18	1	196646753	CFH	575G>T	**Cys192Phe**	NA	1665	752	0.0300	0.0000		D	D	NA	Table 3	NA	NA
21	1	196646753	CFH	575G>T	**Cys192Phe**	NA	-	-	-	-	1 family with 6 carriers	D	D	NA	Table 3	NA	NA
4	1	196646756	CFH	578C>T	**Ser193Leu**	NA	80	-	0.6250	-		D	D	NA	Table 3	NA	NA
7	1	196646756	CFH	578C>T	**Ser193Leu**	NA	1831	1367	0.1365	0.0000	2 families with 5 carriers	D	D	NA	Table 3	NA	NA
14	1	196646758	CFH	580G>A	Asp194Asn	NA	33	27	0.0000	1.8519		T	B	0.0017	None	NA	NA
18	1	196646771	CFH	593G>A	Trp198Ter	NA	1665	752	0.0300	0.0000		NA	NA	NA	None	NA	NA

Source	Chr	Position	Gene	cDNA change (c.)	Protein change (p.)	rs-number	Number Cases	Number Controls	MAF Cases (%)	MAF Controls (%)	Families (incl proband)	SIFT	Poly Phen	ExAC (%)	Function	IAMDGC Odds Ratio	IAMDGC P-Value
19	1	196646785	CFH	607-610 dupCCAA	Lys204 Thrfs*26	NA	21	192	2.3810	0.0000	1 family with 4 carriers	NA	NA	NA	None	NA	NA
18	1	196646794	CFH	616G>A	Val206Met	NA	1665	752	0.0300	0.0000		D	D	NA	None	NA	NA
4	1	196648780	CFH	647T>C	Ile216Thr	rs183474263	80	-	0.6250	-		T	B	0.0085	None	NA	NA
18	1	196648785	CFH	652G>T	Gly218Ter	NA	1665	752	0.0300	0.0000		NA	NA	NA	None	NA	NA
23	1	196648788	CFH	655T>C	Ser219Pro	NA	2335	797	0.0214	0.0000		T	P	NA	None	NA	NA
6	1	196648794	CFH	661A>G	**Ile221Val**	NA	17832	16143	0.0200	0.0000		T	B	0.0025	None	11.80	0.03
18	1	196648794	CFH	661A>G	**Ile221Val**	rs774239374	1665	752	0.0300	0.0000		T	B	0.0025	None	11.80	0.03
23	1	196648794	CFH	661A>G	**Ile221Val**	rs774239374	2335	797	0.0214	0.0000		T	B	0.0025	None	11.80	0.03
18	1	196648828	CFH	965G>A	Arg232Gln	NA	1665	752	0.0601	0.0000		D	D	NA	None	NA	NA
6	1	196648836	CFH	703T>C	**Tyr235His**	NA	17832	16144	0.0000	0.0000		D	D	0.0017	None	3.42	0.44
23	1	196648836	CFH	703T>C	**Tyr235His**	rs754205169	2335	797	0.0214	0.0000		D	D	0.0017	None	3.42	0.44
18	1	196648849	CFH	716T>C	Met239Thr	NA	1665	752	0.0300	0.0000		T	B	NA	None	NA	NA
18	1	196648903	CFH	770G>A	Arg257His	rs140107330	1665	752	0.0000	0.0665		T	B	0.0033	None	NA	NA
6	1	196648906	CFH	773C>T	Pro258Leu	NA	17831	16141	0.0100	0.0000		D	D	0.0008	None	7.95	0.14
18	1	196648924	CFH	**790+1G>A**	**splice site**	NA	1665	752	0.0300	0.0000		NA	NA	NA	Table 3	NA	NA
21	1	196648924	CFH	**790+1G>A**	**splice site**	NA	-	-	-	-	1 family with 2 carriers	NA	NA	NA	Table 3	NA	NA
18	1	196654234	CFH	833C>G	Tyr277Ter	NA	1665	752	0.0300	0.0000		NA	NA	NA	Table 3	NA	NA
23	1	196654274	CFH	871A>T	Thr291Ser	NA	2335	797	0.0214	0.0000		T	B	NA	None	0.37	0.54
4	1	196654304	CFH	901_902 del	Ala301A snfs*25	NA	80	-	0.6250	-		NA	NA	NA	None	NA	NA
6	1	196654310	CFH	907C>T	**Arg303Trp**	rs142937931	17831	16144	0.0100	0.0000		T	D	0.0066	None	12.25	0.04
23	1	196654310	CFH	907C>T	**Arg303Trp**	rs142937931	2335	797	0.0214	0.0000		T	D	0.0066	None	12.25	0.04
6	1	196654311	CFH	908G>A	Arg303Gln	NA	17831	16141	0.0100	0.0000		T	P	0.0017	Table 3	9.47	0.08
14	1	196654345	CFH	942G>T	Trp314Cys	NA	33	27	0.0000	1.8519		D	D	NA	None	NA	NA
18	1	196658559	CFH	974G>A	**Cys325Tyr**	NA	1665	752	0.0300	0.0000		D	D	NA	None	NA	NA
23	1	196658559	CFH	974G>A	**Cys325Tyr**	NA	2335	797	0.0428	0.0000		D	D	NA	None	NA	NA
18	1	196658607	CFH	1022G>A	**Arg341His**	rs371192606	1665	752	0.0300	0.0665		T	B	0.0017	None	NA	NA
23	1	196658607	CFH	1022G>A	**Arg341His**	rs371192606	2335	797	0.0214	0.0000		T	B	0.0017	None	NA	NA

23	1	196658617	CFH	1032C>G	Tyr344Ter	NA	2335	797	0.0214	0.0000		NA	NA	NA	None	NA	NA
18	1	196658676	CFH	1091C>T	Pro364Leu	rs765531414	1665	752	0.0300	0.0000		D	P	0.0008	None	NA	NA
4	1	196658720	CFH	1135T>C	Trp379Arg	NA	80	-	0.6250	-		D	D	NA	None	NA	NA
23	1	196658724	CFH	1139C>A	Ser380Ter	NA	2335	797	0.0214	0.0000		NA	NA	NA	None	NA	NA
18	1	196658736	CFH	1151C>G	**Pro384Arg**	rs747364631	1665	752	0.0300	0.0000		D	P	NA	None	NA	NA
23	1	196658736	CFH	1151C>G	**Pro384Arg**	rs747364631	2335	797	0.0642	0.0000		D	P	NA	None	NA	NA
23	1	196659222	CFH	1189G>T	Gly397Ter	NA	2335	797	0.0214	0.0000		NA	NA	NA	None	3.35	0.45
6	1	196659231	CFH	1198C>A	**Gln400Lys**	rs201671665	17819	16131	0.0200	0.0200		T	B	0.0078	Table 3	0.91	0.87
4	1	196659231	CFH	1198C>A	**Gln400Lys**	rs201671665	80	-	1.2500	-		T	B	0.0078	Table 3	0.91	0.87
18	1	196659231	CFH	1198C>A	**Gln400Lys**	rs201671665	1665	752	0.0901	0.0000		T	B	0.0078	Table 3	0.91	0.87
23	1	196659231	CFH	1198C>A	**Gln400Lys**	rs201671665	2335	797	0.0428	0.0000		T	B	0.0078	Table 3	0.91	0.87
2	1	196659255	CFH	1222C>T	Gln408Ter	rs121913061	30	182	3.3333	0.0000	2 families with 10 carriers	NA	NA	NA	None	NA	NA
18	1	196659261	CFH	1228A>G	Lys410Glu	NA	1665	752	0.0000	0.0665		T	B	NA	None	NA	NA
18	1	196659324	CFH	1291T>A	Cys431Ser	rs121913056	1665	752	0.0300	0.0000		D	D	NA	Table 3	NA	NA
23	1	196659343	CFH	1310C>T	Ser437Phe	NA	2335	797	0.0000	0.0627		T	B	NA	None	NA	NA
23	1	196682885	CFH	1357A>G	Ile453Val	NA	2335	797	0.0000	0.0627		D	B	NA	None	NA	NA
18	1	196682889	CFH	1361A>C	Asp454Ala	NA	1665	752	0.0300	0.0000		T	B	NA	None	NA	NA
6	1	196682932	CFH	1404C>T	Ala468Ala	rs55872061	17819	16137	0.0000	0.0000				0.0084		5.37	0.29
6	1	196682946	CFH	1418C>T	**Ala473Val**	NA	17780	16100	0.0300	0.0300		T	B	0.0083	None	0.93	0.87
18	1	196682946	CFH	1418C>T	**Ala473Val**	rs371053403	1665	752	0.0300	0.0000		T	B	0.0083	None	0.93	0.87
23	1	196682946	CFH	1418C>T	**Ala473Val**	rs371053403	2335	797	0.0428	0.0000		T	B	0.0083	None	0.93	0.87
9	1	196683035	CFH	1507C>G	**Pro503Ala**	rs570523689	1551	1444	0.2579	0.1731	1 family with 4 carriers	D	P	NA	None	NA	NA
18	1	196683035	CFH	1507C>G	**Pro503Ala**	rs570523689	1665	752	0.0300	0.0000		D	P	NA	None	NA	NA
23	1	196683035	CFH	1507C>G	**Pro503Ala**	rs570523689	2335	797	0.0214	0.0000		D	P	NA	None	NA	NA
6	1	196684751	CFH	1548T>A	**Asn516Lys**	rs147403664	17817	16139	0.0600	0.0300		T	D	0.0405	None	2.05	0.07
18	1	196684751	CFH	1548T>A	**Asn516Lys**	rs147403664	1665	752	0.0300	0.0000		T	D	0.0405	None	2.05	0.07
23	1	196684751	CFH	1548T>A	**Asn516Lys**	rs147403664	2335	797	0.0214	0.0000		T	D	0.0405	None	2.05	0.07
23	1	196684784	CFH	1581G>C	Lys527Asn	NA	2335	797	0.0214	0.0000		T	D	NA	None	3.28	0.46
6	1	196684855	CFH	1652T>C	**Ile551Thr**	rs35453854	17832	16144	0.0000	0.0100		T	D	0.5042	None	0.41	0.37
18	1	196684855	CFH	1552T>C	**Ile551Thr**	rs35453854	1665	752	0.0300	0.0000		T	D	0.5042	None	0.41	0.37
23	1	196684855	CFH	1652T>C	**Ile551Thr**	rs35453854	2335	797	0.0857	0.3764		T	D	0.5042	None	0.41	0.37

Source	Chr	Position	Gene	cDNA change (c.)	Protein change (p.)	rs-number	Number Cases	Number Controls	MAF Cases (%)	MAF Controls (%)	Families (incl proband)	SIFT	Poly Phen	ExAC (%)	Function	IAMDGC Odds Ratio	IAMDGC P-Value
18	1	196684888	CFH	1685C>A	**Pro562His**	NA	1665	752	0.0601	0.0000		D	D	NA	None	9.53	0.06
23	1	196684888	CFH	1685C>A	**Pro562His**	NA	2335	797	0.0214	0.0000		D	D	NA	None	9.53	0.06
2	1	196694253	CFH	1699A>G	**Arg567Gly**	rs757756991	30	182	1.6667	0.0000		T	D	0.0009	None	5.11	0.06
18	1	196694253	CFH	1699A>G	**Arg567Gly**	rs757756991	1665	752	0.0601	0.0000		T	D	0.0009	None	5.11	0.06
23	1	196694254	CFH	1700G>A	Arg567Lys	NA	2335	797	0.0000	0.0627		T	B	NA	None	0.38	0.56
6	1	196694299	CFH	1745G>A	**Arg582His**	rs138890387	17832	16144	0.0000	0.0000		T	B	0.0017	None	3.13	0.48
23	1	196694299	CFH	1745G>A	**Arg582His**	rs138890387	2335	797	0.0214	0.0000		T	B	0.0017	None	3.13	0.48
23	1	196694325	CFH	1771G>A	Glu591Lys	NA	2335	797	0.0214	0.0000		T	B	NA	None	NA	NA
6	1	196694379	CFH	1825G>A	**Val609Ile**	rs148165372	17832	16143	0.0500	0.0700		T	B	0.0347	Table 3	0.70	0.29
18	1	196694379	CFH	1825G>A	**Val609Ile**	rs148165372	1665	752	0.0000	0.1995		T	B	0.0347	Table 3	0.70	0.29
23	1	196694379	CFH	1825G>A	**Val609Ile**	rs148165372	2335	797	0.0214	0.0627		T	B	0.0347	Table 3	0.70	0.29
18	1	196694409	CFH	1855G>A	Asp619Asn	NA	1665	752	0.0000	0.0665		T	B	NA	None	NA	NA
23	1	196694418	CFH	1864A>T	Ile622Leu	NA	2335	797	0.0214	0.0000		T	B	NA	None	3.33	0.45
6	1	196694427	CFH	1873G>T	Glu625Ter	rs150694809	17831	16143	0.0000	0.0100		NA	NA	0.0008	None	0.18	0.20
6	1	196695675	CFH	1949G>T	**Gly650Val**	rs143237092	17828	16141	0.0400	0.0300		T	B	0.0232	None	1.52	0.33
17	1	196695675	CFH	1949G>T	**Gly650Val**	rs143237092	1676	745	0.0895	0.0671		T	B	0.0232	None	1.52	0.33
18	1	196695675	CFH	1949G>T	**Gly650Val**	rs143237092	1665	752	0.1201	0.0665		T	B	0.0232	None	1.52	0.33
23	1	196695675	CFH	1949G>T	**Gly650Val**	rs143237092	2335	797	0.0428	0.0000		T	B	0.0232	None	1.52	0.33
6	1	196695724	CFH	1998G>T	**Lys666Asn**	NA	17830	16143	0.0100	0.0000		D	D	0.0008	None	2.59	0.34
23	1	196695724	CFH	1998G>T	**Lys666Asn**	rs752859825	2335	797	0.0214	0.0000		D	D	0.0008	None	2.59	0.34
23	1	196695737	CFH	2011A>T	Ile671Phe	NA	2335	797	0.0214	0.0000		D	D	NA	None	NA	NA
18	1	196695930	CFH	2096A>G	His699Arg	NA	1665	752	0.0300	0.0000		D	D	NA	None	NA	NA
18	1	196695985	CFH	2151C>A	Phe717Leu	rs763441589	1665	752	0.0300	0.0000		D	B	0.0066	None	NA	NA
23	1	196696005	CFH	2171C>A	Thr724Lys	rs142902005	2335	797	0.0000	0.0627		D	P	0.0099	None	NA	NA
6	1	196696029	CFH	2195C>T	**Thr732Met**	rs201360629	17830	16143	0.0000	0.0000		T	B	0.0099	None	1.09	0.94
23	1	196696029	CFH	2195C>T	**Thr732Met**	rs201360629	2335	797	0.0214	0.0000		T	B	0.0099	None	1.09	0.94
6	1	196697644	CFH	2405A>G	Asn802Ser	rs374526857	17832	16144	0.0000	0.0100		T	B	0.0025	None	0.11	0.06
14	1	196705956	CFH	2416G>A	Ala806Thr	rs753331225	33	27	0.0000	1.8519		T	B	NA	None	NA	NA
6	1	196706001	CFH	2461C>T	**His821Tyr**	NA	17832	16144	0.0200	0.0100		T	B	0.0092	None	1.75	0.37
23	1	196706001	CFH	2461C>T	**His821Tyr**	rs367687415	2335	797	0.0214	0.0000		T	B	0.0092	None	1.75	0.37

6	1	196706028	CFH	2488C>T	Arg830Trp	rs62641696	17832	16141	0.0000	0.0100	D	D	0.0083	None	0.51	0.51
6	1	196706659	CFH	2651C>A	**Ser884Tyr**	rs114743644	17832	16143	0.0000	0.0000	D	P	0.0293	None	3.22	0.47
23	1	196706659	CFH	2651C>A	**Ser884Tyr**	rs114743644	2335	797	0.0214	0.0000	D	P	0.0293	None	3.22	0.47
6	1	196706677	CFH	2669G>T	**Ser890Ile**	rs515299	17832	16144	0.2400	0.2200	T	B	1.9940	Table 3	1.03	0.86
17	1	196706677	CFH	2669G>T	**Ser890Ile**	rs515299	1676	745	0.3878	0.4698	T	B	1.9940	Table 3	1.03	0.86
18	1	196706677	CFH	2669G>T	**Ser890Ile**	rs515299	1665	752	0.3604	0.4654	T	B	1.9940	Table 3	1.03	0.86
23	1	196706677	CFH	2669G>T	**Ser890Ile**	rs515299	2335	797	0.5567	0.7528	T	B	1.9940	Table 3	1.03	0.86
6	1	196706683	CFH	2675C>T	**Ala892Val**	rs151068461	17829	16138	0.0200	0.0100	T	B	0.0108	None	1.60	0.47
18	1	196706683	CFH	2675C>T	**Ala892Val**	rs151068461	1665	752	0.0000	0.0665	T	B	0.0108	None	1.60	0.47
23	1	196706683	CFH	2675C>T	**Ala892Val**	rs151068461	2335	797	0.0000	0.0627	T	B	0.0108	None	1.60	0.47
6	1	196709816	CFH	2850G>T	**Gln950His**	rs149474608	17832	16144	0.5100	0.7000	D	P	0.1211	Table 3	0.72	2.58E-03
4	1	196709816	CFH	2850G>T	**Gln950His**	rs149474608	80	-	1.2500	-	D	P	0.3583	Table 3	0.72	2.58E-03
5	1	196709816	CFH	2850G>T	**Gln950His**	rs149474608	12	-	4.1667	-	D	P	0.3583	Table 3	0.72	2.58E-03
14	1	196709816	CFH	2850G>T	**Gln950His**	rs149474608	33	27	3.0303	0.0000	D	P	0.3583	Table 3	0.72	2.58E-03
17	1	196709816	CFH	2850G>T	**Gln950His**	rs149474608	1676	745	0.2685	0.6711	D	P	0.3583	Table 3	0.72	2.58E-03
18	1	196709816	CFH	2850G>T	**Gln950His**	rs149474608	1665	752	0.2703	0.6649	D	P	0.3583	Table 3	0.72	2.58E-03
20	1	196709816	CFH	2850G>T	**Gln950His**	rs149474608	84	-	0.5952	-	D	P	0.3583	Table 3	0.72	2.58E-03
23	1	196709816	CFH	2850G>T	**Gln950His**	rs149474608	2335	797	0.6638	1.1292	D	P	0.3583	Table 3	0.72	2.58E-03
6	1	196709833	CFH	2867C>T	**Thr956Met**	rs145975787	17832	16144	0.2300	0.2200	T	D	0.1211	Table 3	1.04	0.83
4	1	196709833	CFH	2867C>T	**Thr956Met**	rs145975787	80	-	0.6250	-	T	D	0.1211	Table 3	1.04	0.83
17	1	196709833	CFH	2867C>T	**Thr956Met**	rs145975787	1676	745	0.1193	0.4027	T	D	0.1211	Table 3	1.04	0.83
18	1	196709833	CFH	2867C>T	**Thr956Met**	rs145975787	1665	752	0.1201	0.3324	T	D	0.1211	Table 3	1.04	0.83
23	1	196709833	CFH	2867C>T	**Thr956Met**	rs145975787	2335	797	0.2784	0.1882	T	D	0.1211	Table 3	1.04	0.83
6	1	196709845	CFH	2879T>C	**Phe960Ser**	rs115722139	17830	16136	0.0100	0.0100	T	B	0.0049	None	1.40	0.66
23	1	196709845	CFH	2879T>C	**Phe960Ser**	rs115722139	2335	797	0.0214	0.0000	T	B	0.0049	None	1.40	0.66
23	1	196709884	CFH	2918G>A	Cys973Tyr	NA	2335	797	0.0214	0.0000	D	P	NA	None	3.22	0.47
18	1	196709898	CFH	2932T>C	Trp978Arg	NA	1665	752	0.0300	0.0000	D	D	NA	None	NA	NA
18	1	196709907	CFH	2941C>T	Pro981Ser	NA	1665	752	0.0300	0.0000	T	P	NA	None	NA	NA
6	1	196711052	CFH	3004G>C	**Gly1002Arg**	rs201816520	17830	16143	0.0100	0.0200	T	D	0.0107	None	0.44	0.20
18	1	196711052	CFH	3004G>C	**Gly1002Arg**	rs201816520	1665	752	0.0000	0.0665	T	D	0.0107	None	0.44	0.20
6	1	196711067	CFH	3019G>T	**Val1007Leu**	rs534399	17832	16144	0.2400	0.2300	T	B	2.6430	Table 3	0.99	0.96
17	1	196711067	CFH	3019G>T	**Val1007Leu**	rs534399	1676	745	0.4177	0.5369	T	B	2.6430	Table 3	0.99	0.96
18	1	196711067	CFH	3019G>T	**Val1007Leu**	rs534399	1665	752	0.3904	0.4654	T	B	2.6430	Table 3	0.99	0.96

Source	Chr	Position	Gene	cDNA change (c.)	Protein change (p.)	rs-number	Number Cases	Number Controls	MAF Cases (%)	MAF Controls (%)	Families (incl proband)	SIFT	Poly Phen	ExAC (%)	Function	IAMDGC Odds Ratio	IAMDGC P-Value
23	1	196711067	CFH	3019G>T	**Val1007Leu**	rs534399	2335	797	0.7066	1.0665		T	B	2.6430	Table 3	0.99	0.96
23	1	196711076	CFH	3028G>A	Ala1010Thr	rs11539862	2335	797	0.0214	0.0000		T	B	0.0008	None	NA	NA
18	1	196711077	CFH	3029C>T	Ala1010Val	NA	1665	752	0.0300	0.0000		D	B	NA	None	NA	NA
18	1	196711158	CFH	3110G>A	Trp1037Ter	NA	1665	752	0.0300	0.0000		NA	NA	NA	None	NA	NA
6	1	196712596	CFH	3148A>T	**Asn1050Tyr**	rs35274867	17832	16144	0.7400	2.0700		D	B	1.5120	Table 3	0.36	5.92E-44
2	1	196712596	CFH	3148A>T	**Asn1050Tyr**	rs35274867	30	182	1.6667	0.0000		D	B	1.5120	Table 3	0.36	5.92E-44
14	1	196712596	CFH	3148A>T	**Asn1050Tyr**	rs35274867	33	27	1.5152	5.5556		D	B	1.5120	Table 3	0.36	5.92E-44
17	1	196712596	CFH	3148A>T	**Asn1050Tyr**	rs35274867	1676	745	0.6563	1.8121		D	B	1.5120	Table 3	0.36	5.92E-44
18	1	196712596	CFH	3148A>T	**Asn1050Tyr**	rs35274867	1665	752	0.6006	1.7952		D	B	1.5120	Table 3	0.36	5.92E-44
23	1	196712596	CFH	3148A>T	**Asn1050Tyr**	rs35274867	2335	797	1.1563	2.2585		D	B	1.5120	Table 3	0.36	5.92E-44
6	1	196712600	CFH	3152C>T	**Pro1051Leu**	NA	17832	16144	0.0100	0.0100		D	D	0.0033	None	0.87	0.88
18	1	196712600	CFH	3152C>T	**Pro1051Leu**	rs375365236	1665	752	0.0300	0.0000		D	D	0.0033	None	0.87	0.88
6	1	196712616	CFH	3168T>G	**Asn1056Lys**	rs148069859	17829	16143	0.0000	0.0200		D	D	0.0074	None	0.08	0.02
23	1	196712616	CFH	3168T>G	**Asn1056Lys**	rs148069859	2335	797	0.0214	0.0000		D	D	0.0074	None	0.08	0.02
23	1	196712620	CFH	3172T>C	Tyr1058His	rs55679475	2335	797	0.0000	0.0627		T	B	NA	None	NA	NA
6	1	196712624	CFH	3176T>C	**Ile1059Thr**	rs35343172	17832	16144	0.0000	0.0100		T	B	0.6755	None	0.32	0.22
18	1	196712624	CFH	3176T>C	**Ile1059Thr**	rs35343172	1665	752	0.0300	0.0000		T	B	0.6755	None	0.32	0.22
23	1	196712624	CFH	3176T>C	**Ile1059Thr**	rs35343172	2335	797	0.1499	0.3764		T	B	0.6755	None	0.32	0.22
23	1	196712626	CFH	3178G>C	Val1060Leu	rs55771831	2335	797	0.0000	0.0627		T	B	NA	None	NA	NA
18	1	196712674	CFH	3226C>G	**Gln1076Glu**	rs62625015	1665	752	0.0601	0.0000		T	B	NA	Table 3	NA	NA
23	1	196712674	CFH	3226C>G	**Gln1076Glu**	rs62625015	2335	797	0.0428	0.0627		T	B	NA	Table 3	NA	NA
2	1	196712682	CFH	3234G>T	Arg1078Ser	rs121913062	30	182	1.6667	0.0000	1 family with 3 carriers	T	B	0.0058	None	NA	NA
14	1	196712713	CFH	3265_3266insA	Val1089 Aspfs*28	NA	33	27	1.5152	0.0000		NA	NA	NA	None	NA	NA
6	1	196715063	CFH	3427C>G	**Gln1143Glu**	rs34247141	17832	16144	0.0000	0.0200		T	B	0.9658	None	0.21	0.06
18	1	196715063	CFH	3427C>G	**Gln1143Glu**	rs34247141	1665	752	0.0300	0.0665		T	B	0.9658	None	0.21	0.06
23	1	196715063	CFH	3427C>G	**Gln1143Glu**	rs34247141	2335	797	0.1927	0.4391		T	B	0.9658	None	0.21	0.06
23	1	196716244	CFH	3497C>A	Pro1166Gln	NA	2335	797	0.0214	0.0000		D	B	NA	None	NA	NA
6	1	196716328	CFH	3581G>A	**Gly1194Asp**	NA	17832	16144	0.0100	0.0000		T	B	0.0033	Table 3	7.41	0.12
17	1	196716328	CFH	3581G>A	**Gly1194Asp**	rs761877050	1676	745	0.1492	0.0000		T	B	0.0033	Table 3	7.41	0.12

18	1	196716328	CFH	3581G>A	**Gly1194Asp**	rs761877050	1665	752	0.1502	0.0000		T	B	0.0033	Table 3	7.41	0.12
23	1	196716328	CFH	3581G>A	**Gly1194Asp**	rs761877050	2335	797	0.0214	0.0000		T	B	0.0033	Table 3	7.41	0.12
23	1	196716337	CFH	3590T>C	Val1197Ala	rs460184	2335	797	0.0214	0.0000		D	D	NA	Table 3	NA	NA
23	1	196716353	CFH	3606A>C	Lys1202Asn	rs147478315	2335	797	0.0214	0.0000		T	P	NA	None	2.46	0.41
6	1	196716354	CFH	3607C>T	Arg1203Trp	rs145347741	17832	16144	0.0000	0.0000		T	B	0.0082	Table 3	0.68	0.82
18	1	196716364	CFH	3617G>A	Arg1206His	NA	1665	752	0.0300	0.0000		T	B	NA	None	NA	NA
6	1	196716375	CFH	3628C>T	**Arg1210Cys**	rs121913059	17832	16144	0.3200	0.0100		T	B	0.0173	Table 3	20.28	8.91E-24
4	1	196716375	CFH	3628C>T	**Arg1210Cys**	rs121913059	80	-	1.2500	-		T	B	0.0173	Table 3	20.28	8.91E-24
11	1	196716375	CFH	3628C>T	**Arg1210Cys**	rs121913059	1364	1208	0.0367	0.0000		T	B	0.0173	Table 3	20.28	8.91E-24
14	1	196716375	CFH	3628C>T	**Arg1210Cys**	rs121913059	2414	1120	0.6835	0.0446	7 carrier first-degree relatives	T	B	0.0173	Table 3	20.28	8.91E-24
15	1	196716375	CFH	3628C>T	**Arg1210Cys**	rs121913059	259	330	0.9653	0.0000	2 families with 5 carriers	T	B	0.0173	Table 3	20.28	8.91E-24
16	1	196716375	CFH	3628C>T	**Arg1210Cys**	rs121913059	1589	1386	0.0000	0.0000		T	B	0.0173	Table 3	20.28	8.91E-24
17	1	196716375	CFH	3628C>T	**Arg1210Cys**	rs121913059	1676	745	0.4773	0.0671		T	B	0.0173	Table 3	20.28	8.91E-24
18	1	196716375	CFH	3628C>T	**Arg1210Cys**	rs121913059	1665	752	0.4805	0.0665		T	B	0.0173	Table 3	20.28	8.91E-24
20	1	196716375	CFH	3628C>T	**Arg1210Cys**	rs121913059	84	-	0.5952	-		T	B	0.0173	Table 3	20.28	8.91E-24
23	1	196716375	CFH	3628C>T	**Arg1210Cys**	rs121913059	2335	797	0.4925	0.0000		T	B	0.0173	Table 3	20.28	8.91E-24
18	1	196716427	CFH	3680C>T	Thr1227Ile	rs773524517	1665	752	0.0300	0.0000		T	P	0.0025	None	NA	NA
10	4	110662063	CFI	1738C>T	**Gln580Ter**	rs775030152	2266	1400	0.0221	0.0000		NA	NA	0.0008	None	NA	NA
17	4	110662063	CFI	1738C>T	**Gln580Ter**	rs775030152	1712	781	0.0292	0.0000		NA	NA	0.0008	None	NA	NA
10	4	110662068	CFI	1733T>C	**Ile578Thr**	rs760148430	2266	1400	0.0441	0.0000		D	D	0.0025	None	NA	NA
17	4	110662068	CFI	1733T>C	**Ile578Thr**	rs760148430	1712	781	0.0292	0.0000		D	D	0.0025	None	NA	NA
23	4	110662068	CFI	1733T>C	**Ile578Thr**	rs760148430	2335	797	0.0428	0.0000		D	D	0.0025	None	NA	NA
23	4	110662092	CFI	1709G>C	Ser570Thr	rs200973120	2335	797	0.0214	0.0000		T	D	0.0041	None	NA	NA
10	4	110662140	CFI	1661A>T	**Glu554Val**	rs754572081	2266	1400	0.0662	0.0357		T	D	0.0016	None	NA	NA
17	4	110662140	CFI	1661A>T	**Glu554Val**	rs754572081	1712	781	0.0876	0.0000		T	D	0.0016	None	NA	NA
7	4	110662144	CFI	1657C>T	**Pro553Ser**	rs113460688	1831	1367	0.2731	0.0732	1 family with 3 carriers	T	B	0.1270	Table 3	NA	NA
10	4	110662144	CFI	1657C>T	**Pro553Ser**	rs113460688	2266	1400	0.5737	0.2143		T	B	0.1270	Table 3	NA	NA
17	4	110662144	CFI	1657C>T	**Pro553Ser**	rs113460688	1712	781	0.5257	0.1280		T	B	0.1270	Table 3	NA	NA

Source	Chr	Position	Gene	cDNA change (c.)	Protein change (p.)	rs-number	Number Cases	Number Controls	MAF Cases (%)	MAF Controls (%)	Families (incl proband)	SIFT	Poly Phen	ExAC (%)	Function	IAMDGC Odds Ratio	IAMDGC P-Value
23	4	110662144	CFI	1657C>T	**Pro553Ser**	rs113460688	2335	797	0.2784	0.2509		T	B	0.1270	Table 3	NA	NA
23	4	110662159	CFI	1642G>C	Glu548Gln	rs7437875	2335	797	0.0214	0.0000		T	D	0.0717	None	NA	NA
10	4	110662173	CFI	1628T>C	**Val543Ala**	NA	2266	1400	0.0221	0.0000		D	P	NA	None	NA	NA
17	4	110662173	CFI	1628T>C	**Val543Ala**	NA	1712	781	0.0292	0.0000		D	P	NA	None	NA	NA
23	4	110662177	CFI	1624G>A	Gly542Ser	rs747755806	2335	797	0.0214	0.0000		T	D	0.0008	None	NA	NA
10	4	110662179	CFI	1622G>A	**Trp541Ter**	NA	2266	1400	0.0221	0.0000		NA	NA	NA	None	NA	NA
17	4	110662179	CFI	1622G>A	**Trp541Ter**	NA	1712	781	0.0292	0.0000		NA	NA	NA	None	NA	NA
10	4	110662193	CFI	1608T>A	Asn536Lys	NA	2266	1400	0.0000	0.0357		T	D	NA	None	NA	NA
6	4	110663647	CFI	1534G>A	**Gly512Ser**	rs770828632	17832	16144	0.0100	0.0000		D	D	0.0017	None	5.49	0.23
10	4	110663647	CFI	1534G>A	**Gly512Ser**	rs770828632	2266	1400	0.0221	0.0000		D	D	0.0017	None	5.49	0.23
17	4	110663647	CFI	1534G>A	**Gly512Ser**	rs770828632	1712	781	0.0292	0.0000		D	D	0.0017	None	5.49	0.23
23	4	110663647	CFI	1534G>A	**Gly512Ser**	rs770828632	2335	797	0.0214	0.0000		D	D	0.0017	None	5.49	0.23
10	4	110663677	CFI	1504C>T	**Arg502Cys**	NA	2266	1400	0.0221	0.0000		D	D	NA	None	NA	NA
17	4	110663677	CFI	1504C>T	**Arg502Cys**	NA	1712	781	0.0292	0.0000		D	D	NA	None	NA	NA
10	4	110663683	CFI	1498G>A	Gly500Arg	rs558749473	2266	1400	0.0221	0.0000		T	B	0.0033	None	NA	NA
10	4	110663707	CFI	1474A>C	**Ile492Leu**	rs200025458	2266	1400	0.0221	0.0000		T	B	0.0025	None	NA	NA
17	4	110663707	CFI	1474A>C	**Ile492Leu**	rs200025458	1712	781	0.0292	0.0000		T	B	0.0025	None	NA	NA
10	4	110663722	CFI	1459G>T	**Gly487Cys**	NA	2266	1400	0.0221	0.0000		D	D	NA	None	NA	NA
17	4	110663722	CFI	1459G>T	**Gly487Cys**	NA	1712	781	0.0292	0.0000		D	D	NA	None	NA	NA
10	4	110667377	CFI	**1429+1C>G**	Splice site	rs368555424	2266	1400	0.0883	0.0000		NA	NA	0.0033	None	NA	NA
17	4	110667377	CFI	**1429+1C>G**	Splice site	rs368555424	1712	781	0.0876	0.0000		NA	NA	0.0033	None	NA	NA
23	4	110667377	CFI	**1429+1C>G**	Splice site	rs368555424	2335	797	0.0857	0.0000		NA	NA	0.0033	None	NA	NA
6	4	110667378	CFI	1429G>C	**Asp477His**	NA	17832	16144	0.0100	0.0000		D	P	0.0033	None	10.26	0.05
10	4	110667378	CFI	1429G>C	**Asp477His**	rs754972981	2266	1400	0.0441	0.0000		D	P	0.0033	None	10.26	0.05
17	4	110667378	CFI	1429G>C	**Asp477His**	rs754972981	1712	781	0.0584	0.0000		D	P	0.0033	None	10.26	0.05
23	4	110667378	CFI	1429G>C	**Asp477His**	rs754972981	2335	797	0.0428	0.0000		D	P	0.0033	None	10.26	0.05
6	4	110667386	CFI	1421G>A	**Arg474Gln**	NA	17730	16088	0.0200	0.0000		T	P	0.0025	Table 3	4.06	0.12
10	4	110667386	CFI	1421G>A	**Arg474Gln**	rs765956155	2266	1400	0.0221	0.0000		T	P	0.0025	Table 3	4.06	0.12
23	4	110667386	CFI	1421G>A	**Arg474Gln**	rs765956155	2335	797	0.0000	0.0627		T	P	0.0025	Table 3	4.06	0.12
6	4	110667387	CFI	1420C>T	**Arg474Ter**	rs121964913	17831	16143	0.0100	0.0100		NA	NA	0.0050	Table 3	1.07	0.95

10	4	110667387	CFI	1420C>T	**Arg474Ter**	rs121964913	2266	1400	0.0441	0.0000		NA	NA	0.0050	Table 3	1.07	0.95
17	4	110667387	CFI	1420C>T	**Arg474Ter**	rs121964913	1712	781	0.0292	0.0000		NA	NA	0.0050	Table 3	1.07	0.95
10	4	110667408	CFI	1399T>C	**Cys467Arg**	NA	2266	1400	0.0221	0.0000		D	D	NA	None	NA	NA
17	4	110667408	CFI	1399T>C	**Cys467Arg**	NA	1712	781	0.0292	0.0000		D	D	NA	None	NA	NA
6	4	110667421	CFI	1386A>T	**Gln462His**	rs143827877	17832	16144	0.0100	0.0100		T	P	0.0124	None	0.60	0.53
10	4	110667421	CFI	1386A>T	**Gln462His**	rs143827877	2266	1400	0.0000	0.0357		T	P	0.0124	None	0.60	0.53
10	4	110667431	CFI	1376A>C	**Tyr459Ser**	NA	2266	1400	0.0221	0.0000		T	P	NA	Table 3	2.38	0.27
17	4	110667431	CFI	1376A>C	**Tyr459Ser**	NA	1712	781	0.0292	0.0000		T	P	NA	Table 3	2.38	0.27
23	4	110667431	CFI	1376A>C	**Tyr459Ser**	NA	2335	797	0.0214	0.0000		T	P	NA	Table 3	2.38	0.27
6	4	110667485	CFI	1322A>G	**Lys441Arg**	rs41278047	17832	16144	0.5600	0.4700		T	B	0.3421	Table 3	1.15	0.24
10	4	110667485	CFI	1322A>G	**Lys441Arg**	rs41278047	2266	1400	0.6620	0.4643		T	B	0.3421	Table 3	1.15	0.24
17	4	110667485	CFI	1322A>G	**Lys441Arg**	rs41278047	1712	781	0.6717	0.1921		T	B	0.3421	Table 3	1.15	0.24
23	4	110667485	CFI	1322A>G	**Lys441Arg**	rs41278047	2335	797	0.5567	0.2509		T	B	0.3421	Table 3	1.15	0.24
23	4	110667516	CFI	1291G>A	Ala431Thr	rs758017357	2335	797	0.0428	0.0000		D	D	0.0025	Table 3	NA	NA
23	4	110667520	CFI	1287C>G	Asp429Glu	NA	2335	797	0.0214	0.0000		T	D	NA	None	3.36	0.45
10	4	110667554	CFI	1253A>T	**His418Leu**	rs121964912	2266	1400	0.0441	0.0000		D	D	0.0008	Table 3	NA	NA
17	4	110667554	CFI	1253A>T	**His418Leu**	rs121964912	1712	781	0.0584	0.0000		D	D	0.0008	Table 3	NA	NA
6	4	110667561	CFI	1246A>C	**Ile416Leu**	rs61733901	17832	16144	0.0100	0.0000		T	B	0.1113	Table 3	2.23	0.47
10	4	110667561	CFI	1246A>C	**Ile416Leu**	rs61733901	2266	1400	0.0221	0.0000		T	B	0.1113	Table 3	2.23	0.47
17	4	110667561	CFI	1246A>C	**Ile416Leu**	rs61733901	1712	781	0.0292	0.0000		T	B	0.1113	Table 3	2.23	0.47
13	4	110667573	CFI	1234G>A	Val412Met	rs371432629	12	200	0.0000	2.5000	2 families with 7 carriers	D	D	0.0107	None	NA	NA
6	4	110667574	CFI	1233C>A	**Tyr411Ter**	NA	17832	16144	0.0200	0.0100		NA	NA	NA	None	1.83	0.33
23	4	110667574	CFI	1233C>A	**Tyr411Ter**	NA	2335	797	0.0214	0.0000		NA	NA	NA	None	1.83	0.33
10	4	110667590	CFI	1217G>A	**Arg406His**	rs74817407	2266	1400	0.0221	0.2143		D	P	1.6880	Table 3	1.25	0.47
17	4	110667590	CFI	1217G>A	**Arg406His**	rs74817407	1712	781	0.0292	0.0000		D	P	1.6880	Table 3	1.25	0.47
23	4	110667590	CFI	1217G>A	**Arg406His**	rs74817407	2335	797	0.0857	0.1255		D	P	1.6880	Table 3	1.25	0.47
6	4	110667591	CFI	1216C>T	Arg406Cys	rs181729783	17831	16144	0.0100	0.0100		T	D	0.0058	None	1.05	0.96
6	4	110667600	CFI	1207G>A	**Asp403Asn**	rs139881195	17832	16144	0.0100	0.0000		T	B	0.0025	None	1.69	0.63
10	4	110667600	CFI	1207G>A	**Asp403Asn**	rs139881195	2266	1400	0.0221	0.0000		T	B	0.0025	None	1.69	0.63
17	4	110667600	CFI	1207G>A	**Asp403Asn**	rs139881195	1712	781	0.0292	0.0000		T	B	0.0025	None	1.69	0.63
23	4	110667600	CFI	1207G>A	**Asp403Asn**	rs139881195	2335	797	0.0214	0.0000		T	B	0.0025	None	1.69	0.63

Source	Chr	Position	Gene	cDNA change (c.)	Protein change (p.)	rs-number	Number Cases	Number Controls	MAF Cases (%)	MAF Controls (%)	Families (incl proband)	SIFT	Poly Phen	ExAC (%)	Function	IAMDGC Odds Ratio	IAMDGC P-Value
10	4	110667612	CFI	1195T>C	**Trp399Arg**	rs778871974	2266	1400	0.0221	0.0000		T	B	0.0025	None	NA	NA
17	4	110667612	CFI	1195T>C	**Trp399Arg**	rs778871974	1712	781	0.0584	0.0000		T	B	0.0025	None	NA	NA
10	4	110667641	CFI	1166G>A	**Arg389His**	rs777190530	2266	1400	0.0221	0.0000		T	B	0.0017	None	NA	NA
17	4	110667641	CFI	1166G>A	**Arg389His**	rs777190530	1712	781	0.0292	0.0000		T	B	0.0017	None	NA	NA
10	4	110670400	CFI	1122G>T	**Trp374Cys**	NA	2266	1400	0.0221	0.0000		D	D	NA	None	NA	NA
17	4	110670400	CFI	1122G>T	**Trp374Cys**	NA	1712	781	0.0292	0.0000		D	D	NA	None	NA	NA
10	4	110670416	CFI	1106A>C	**Tyr369Ser**	rs776596901	2266	1400	0.0221	0.0000		D	D	0.0008	Table 3	NA	NA
17	4	110670416	CFI	1106A>C	**Tyr369Ser**	rs776596901	1712	781	0.0292	0.0000		D	D	0.0008	Table 3	NA	NA
10	4	110670437	CFI	1085G>C	**Gly362Ala**	rs200619905	2266	1400	0.0000	0.0357		T	B	0.0008	None	NA	NA
17	4	110670437	CFI	1085G>C	**Gly362Ala**	rs200619905	1712	781	0.0000	0.0640		T	B	0.0008	None	NA	NA
6	4	110670451	CFI	1071T>G	Ile357Met	rs200881135	17832	16144	0.0100	0.0000		D	D	0.0033	None	9.39	0.07
10	4	110670456	CFI	1066G>C	Ala356Pro	rs781498531	2266	1400	0.0221	0.0000		D	D	0.0016	None	NA	NA
10	4	110670459	CFI	1063G>A	**Val355Met**	NA	2266	1400	0.0000	0.0357		D	D	NA	None	2.54	0.35
17	4	110670459	CFI	1063G>A	**Val355Met**	NA	1712	781	0.0000	0.0640		D	D	NA	None	2.54	0.35
23	4	110670459	CFI	1063G>A	**Val355Met**	NA	2335	797	0.0428	0.0000		D	D	NA	None	2.54	0.35
23	4	110670674	CFI	1025G>A	Gly342Glu	NA	2335	797	0.0214	0.0000		D	D	NA	None	3.38	0.45
6	4	110670680	CFI	1019T>C	**Ile340Thr**	rs769419740	17829	16135	0.0300	0.0200		D	D	0.0041	Table 3	1.68	0.33
10	4	110670680	CFI	1019T>C	**Ile340Thr**	rs769419740	2266	1400	0.0221	0.0000		D	D	0.0041	Table 3	1.68	0.33
17	4	110670680	CFI	1019T>C	**Ile340Thr**	rs769419740	1712	781	0.0292	0.0000		D	D	0.0041	Table 3	1.68	0.33
23	4	110670680	CFI	1019T>C	**Ile340Thr**	rs769419740	2335	797	0.0214	0.0000		D	D	0.0041	Table 3	1.68	0.33
6	4	110670683	CFI	1016G>A	**Arg339Gln**	NA	17832	16144	0.0200	0.0000		D	D	0.0016	Table 3	11.83	0.03
10	4	110670683	CFI	1016G>A	**Arg339Gln**	rs773085612	2266	1400	0.0221	0.0000		D	D	0.0016	Table 3	11.83	0.03
23	4	110670683	CFI	1016G>A	**Arg339Gln**	rs773085612	2335	797	0.0214	0.0000		D	D	0.0016	Table 3	11.83	0.03
6	4	110670684	CFI	1015C>T	Arg339Ter	NA	17831	16143	0.0100	0.0000		NA	NA	0.0025	None	9.63	0.06
6	4	110670717	CFI	982G>A	**Gly328Arg**	rs144164794	17832	16144	0.0100	0.0000		D	D	0.0115	None	5.67	0.22
10	4	110670717	CFI	982G>A	**Gly328Arg**	rs144164794	2266	1400	0.0221	0.0000		D	D	0.0115	None	5.67	0.22
23	4	110670717	CFI	982G>A	**Gly328Arg**	rs144164794	2335	797	0.0428	0.0000		D	D	0.0115	None	5.67	0.22
6	4	110670749	CFI	950G>A	**Arg317Gln**	NA	17832	16144	0.0000	0.0000		T	B	0.0008	Table 3	2.81	0.52
10	4	110670749	CFI	950G>A	**Arg317Gln**	rs751111134	2266	1400	0.0221	0.0000		T	B	0.0008	Table 3	2.81	0.52
17	4	110670749	CFI	950G>A	**Arg317Gln**	rs751111134	1712	781	0.0292	0.0000		T	B	0.0008	Table 3	2.81	0.52

23	4	110670749	CFI	950G>A	**Arg317Gln**	rs751111134	2335	797	0.0214	0.0000	T	B	0.0008	Table 3	2.81	0.52
6	4	110670750	CFI	949C>T	**Arg317Trp**	rs121964917	17786	16110	0.0500	0.0000	D	B	0.0099	Table 3	12.20	1.97E-04
10	4	110670750	CFI	949C>T	**Arg317Trp**	rs121964917	2266	1400	0.0441	0.0357	D	B	0.0099	Table 3	12.20	1.97E-04
17	4	110670750	CFI	949C>T	**Arg317Trp**	rs121964917	1712	781	0.0584	0.0640	D	B	0.0099	Table 3	12.20	1.97E-04
23	4	110670750	CFI	949C>T	**Arg317Trp**	rs121964917	2335	797	0.0214	0.0000	D	B	0.0099	Table 3	12.20	1.97E-04
10	4	110673634	CFI	930C>A	**Asp310Glu**	NA	2266	1400	0.0441	0.0000	T	P	NA	None	NA	NA
17	4	110673634	CFI	930C>A	**Asp310Glu**	NA	1712	781	0.0292	0.0000	T	P	NA	None	NA	NA
6	4	110673648	CFI	916A>G	Ile306Val	rs113273712	17832	16144	0.0000	0.0000	T	B	0.0480	None	2.42	0.59
10	4	110678925	CFI	898G>A	**Ala300Thr**	rs11098044	2266	1400	0.1103	0.1429	T	B	0.9400	Table 3	2.67	0.01
17	4	110678925	CFI	898G>A	**Ala300Thr**	rs11098044	1712	781	0.1168	0.1280	T	B	0.9400	Table 3	2.67	0.01
23	4	110678925	CFI	898G>A	**Ala300Thr**	rs11098044	2335	797	0.1713	0.3137	T	B	0.9400	Table 3	2.67	0.01
6	4	110678925	CFI	898G>A	**Ala300Thr**	rs11098044	17832	16144	0.0700	0.0200	T	B	0.9400	Table 3	2.67	0.01
6	4	110681450	CFI	859G>A	**Gly287Arg**	rs182078921	17830	16143	0.0400	0.0100	D	D	0.0033	None	4.61	7.61E-03
10	4	110681450	CFI	859G>A	**Gly287Arg**	rs182078921	2266	1400	0.0662	0.0000	D	D	0.0033	None	4.61	7.61E-03
17	4	110681450	CFI	859G>A	**Gly287Arg**	rs182078921	1712	781	0.0876	0.0000	D	D	0.0033	None	4.61	7.61E-03
10	4	110681470	CFI	839G>A	**Gly280Asp**	NA	2266	1400	0.0221	0.0000	D	P	NA	None	NA	NA
17	4	110681470	CFI	839G>A	**Gly280Asp**	NA	1712	781	0.0292	0.0000	D	P	NA	None	NA	NA
23	4	110681508	CFI	801A>C	Lys267Asn	NA	2335	797	0.0214	0.0000	T	P	NA	None	NA	NA
10	4	110681521	CFI	788G>T	**Gly263Val**	rs200544168	2266	1400	0.0441	0.0000	T	D	NA	None	1.92	0.43
17	4	110681521	CFI	788G>T	**Gly263Val**	rs200544168	1712	781	0.0292	0.0000	T	D	NA	None	1.92	0.43
23	4	110681521	CFI	788G>T	**Gly263Val**	rs200544168	2335	797	0.0214	0.0000	T	D	NA	None	1.92	0.43
6	4	110681527	CFI	782G>A	**Gly261Asp**	rs112534524	17832	16144	0.2500	0.2900	T	B	0.1326	Table 3	0.86	0.34
10	4	110681527	CFI	782G>A	**Gly261Asp**	rs112534524	2266	1400	0.2648	0.2857	T	B	0.1326	Table 3	0.86	0.34
17	4	110681527	CFI	782G>A	**Gly261Asp**	rs112534524	1712	781	0.2629	0.1921	T	B	0.1326	Table 3	0.86	0.34
23	4	110681527	CFI	782G>A	**Gly261Asp**	rs112534524	2335	797	0.3426	0.3137	T	B	0.1326	Table 3	0.86	0.34
6	4	110681679	CFI	772G>A	**Ala258Thr**	rs199688124	17832	16144	0.1100	0.0300	T	P	0.0107	Table 3	3.88	6.25E-05
10	4	110681679	CFI	772G>A	**Ala258Thr**	rs199688124	2266	1400	0.0883	0.0000	T	P	0.0107	None	3.88	6.25E-05
17	4	110681679	CFI	772G>A	**Ala258Thr**	rs199688124	1712	781	0.0876	0.0000	T	P	0.0107	None	3.88	6.25E-05
23	4	110681679	CFI	772G>A	**Ala258Thr**	rs199688124	2335	797	0.0857	0.0000	T	P	0.0107	Table 3	3.88	6.25E-05
10	4	110681708	CFI	743G>A	**Gly248Glu**	NA	2266	1400	0.0221	0.0000	D	D	NA	None	NA	NA
17	4	110681708	CFI	743G>A	**Gly248Glu**	NA	1712	781	0.0292	0.0000	D	D	NA	None	NA	NA
10	4	110681732	CFI	719C>G	**Ala240Gly**	rs146444258	2266	1400	0.2648	0.0357	T	D	0.0272	Table 3	NA	NA
17	4	110681732	CFI	719C>G	**Ala240Gly**	rs146444258	1712	781	0.3505	0.0640	T	D	0.0272	Table 3	NA	NA

Source	Chr	Position	Gene	cDNA change (c.)	Protein change (p.)	rs-number	Number Cases	Number Controls	MAF Cases (%)	MAF Controls (%)	Families (incl proband)	SIFT	Poly Phen	ExAC (%)	Function	IAMDGC Odds Ratio	IAMDGC P-Value
23	4	110681732	CFI	719C>G	**Ala240Gly**	rs146444258	2335	797	0.1713	0.0627		T	D	0.0272	Table 3	NA	NA
10	4	110681763	CFI	688G>A	**Val230Met**	NA	2266	1400	0.0221	0.0000		D	D	NA	None	NA	NA
17	4	110681763	CFI	688G>A	**Val230Met**	NA	1712	781	0.0292	0.0000		D	D	NA	None	NA	NA
10	4	110681766	CFI	685T>C	**Cys229Arg**	NA	2266	1400	0.0221	0.0000		D	D	NA	None	NA	NA
17	4	110681766	CFI	685T>C	**Cys229Arg**	NA	1712	781	0.0292	0.0000		D	D	NA	None	NA	NA
10	4	110681781	CFI	670G>A	**Asp224Asn**	rs190001845	2266	1400	0.0221	0.0000		T	B	NA	None	NA	NA
17	4	110681781	CFI	670G>A	**Asp224Asn**	rs190001845	1712	781	0.0292	0.0000		T	B	NA	None	NA	NA
10	4	110681789	CFI	662C>A	**Ser221Tyr**	rs377528991	2266	1400	0.0221	0.0000		T	P	NA	None	NA	NA
17	4	110681789	CFI	662C>A	**Ser221Tyr**	rs377528991	1712	781	0.0292	0.0000		T	P	NA	None	NA	NA
10	4	110682680	CFI	651G>C	**Gln217His**	rs767216603	2266	1400	0.0000	0.0357		T	P	NA	None	NA	NA
17	4	110682680	CFI	651G>C	**Gln217His**	rs767216603	1712	781	0.0000	0.0640		T	P	NA	None	NA	NA
10	4	110682715	CFI	206T>A	**Tyr206Asn**	rs371623439	2266	1400	0.0441	0.0000		T	B	0.0132	None	NA	NA
17	4	110682715	CFI	206T>A	**Tyr206Asn**	rs371623439	1712	781	0.0292	0.0000		T	B	0.0132	None	NA	NA
6	4	110682723	CFI	608C>T	**Thr203Ile**	rs138346388	17831	16143	0.0600	0.0200		T	B	0.0519	None	2.46	0.03
10	4	110682723	CFI	608C>T	**Thr203Ile**	rs138346388	2266	1400	0.0441	0.0000		T	B	0.0519	None	2.46	0.03
17	4	110682723	CFI	608C>T	**Thr203Ile**	rs138346388	1712	781	0.0584	0.0000		T	B	0.0519	None	2.46	0.03
23	4	110682723	CFI	608C>T	**Thr203Ile**	rs138346388	2335	797	0.0214	0.0000		T	B	0.0519	None	2.46	0.03
10	4	110682726	CFI	605G>T	**Arg202Ile**	rs149215929	2266	1400	0.1103	0.2500		T	P	NA	Table 3	NA	NA
17	4	110682726	CFI	605G>T	**Arg202Ile**	rs149215929	1712	781	0.0000	0.0640		T	P	NA	Table 3	NA	NA
10	4	110682739	CFI	594T>C	**Phe198Leu**	NA	2266	1400	0.0221	0.0000		T	B	NA	None	NA	NA
17	4	110682739	CFI	594T>C	**Phe198Leu**	NA	1712	781	0.0292	0.0000		T	B	NA	None	NA	NA
1	4	110682769	CFI	563G>C	**Gly188Ala**	NA	521	627	0.0960	0.0797		D	D	NA	Table 3	NA	NA
20	4	110682769	CFI	563G>C	**Gly188Ala**	NA	809	254	0.0000	0.0000	1 family with 3 carriers	D	D	NA	Table 3	NA	NA
6	4	110682771	CFI	560G>A	**Arg187Gln**	rs143366614	17831	16144	0.0200	0.0100		T	P	0.0124	None	1.30	0.69
10	4	110682771	CFI	560G>A	**Arg187Gln**	rs143366614	2266	1400	0.0221	0.0000		T	P	0.0124	None	1.30	0.69
17	4	110682771	CFI	560G>A	**Arg187Gln**	rs143366614	1712	781	0.0292	0.0000		T	P	0.0124	None	1.30	0.69
6	4	110682772	CFI	559C>T	**Arg187Ter**	NA	17832	16144	0.0200	0.0000		NA	NA	0.0016	None	13.63	0.02
23	4	110682772	CFI	559C>T	**Arg187Ter**	rs368615806	2335	797	0.0428	0.0000		NA	NA	0.0016	None	13.63	0.02
10	4	110682781	CFI	550G>A	**Val184Met**	rs200418129	2266	1400	0.0000	0.0357		T	P	NA	None	NA	NA

17	4	110682781	CFI	550G>A	**Val184Met**	rs200418129	1712	781	0.0000	0.0640		T	P	NA	None	NA	NA
6	4	110682783	CFI	548A>G	His183Arg	rs75612300	17832	16144	0.0000	0.0000		T	B	0.0626	None	2.58	0.56
10	4	110682801	CFI	530A>T	**Asn177Ile**	rs753060374	2266	1400	0.0221	0.0000		T	B	0.0049	None	NA	NA
17	4	110682801	CFI	530A>T	**Asn177Ile**	rs753060374	1712	781	0.0292	0.0000		T	B	0.0049	None	NA	NA
6	4	110682846	CFI	485G>A	**Gly162Asp**	NA	17832	16144	0.0300	0.0000		D	D	0.0008	None	20.29	2.31E-03
10	4	110682846	CFI	485G>A	**Gly162Asp**	rs546607673	2266	1400	0.0883	0.0000		D	D	0.0008	None	20.29	2.31E-03
23	4	110682846	CFI	485G>A	**Gly162Asp**	rs546607673	2335	797	0.0214	0.0000		D	D	0.0008	None	20.29	2.31E-03
6	4	110685721	CFI	454G>A	**Val152Met**	NA	17830	16140	0.0500	0.0100		D	D	0.0025	None	7.57	4.65E-04
10	4	110685721	CFI	454G>A	**Val152Met**	rs367677199	2266	1400	0.0441	0.0000		D	D	0.0025	None	7.57	4.65E-04
17	4	110685721	CFI	454G>A	**Val152Met**	rs367677199	1712	781	0.0584	0.0000		D	D	0.0025	None	7.57	4.65E-04
23	4	110685721	CFI	454G>A	**Val152Met**	rs367677199	2335	797	0.0428	0.0000		D	D	0.0025	None	7.57	4.65E-04
6	4	110685723	CFI	452A>G	**Asn151Ser**	NA	17667	16010	0.0200	0.0300		T	D	0.0008	Table 3	0.74	0.53
23	4	110685723	CFI	452A>G	**Asn151Ser**	rs772044176	2335	797	0.0214	0.0000		T	D	0.0008	Table 3	0.74	0.53
7	4	110685783	CFI	392T>G	Leu131Arg	NA	1831	1367	0.1092	0.0000	1 family with 5 carriers	D	D	NA	Table 3	NA	NA
6	4	110685789	CFI	386T>G	**Val129Gly**	NA	17831	16144	0.0000	0.0000		D	D	NA	None	0.35	0.52
23	4	110685789	CFI	386T>G	**Val129Gly**	NA	2335	797	0.0000	0.0627		D	D	NA	None	0.35	0.52
10	4	110685795	CFI	380T>C	**Val127Ala**	NA	2266	1400	0.0221	0.0000		T	P	NA	None	3.37	0.45
17	4	110685795	CFI	380T>C	**Val127Ala**	NA	1712	781	0.0292	0.0000		T	P	NA	None	3.37	0.45
23	4	110685795	CFI	380T>C	**Val127Ala**	NA	2335	797	0.0214	0.0000		T	P	NA	None	3.37	0.45
10	4	110685802	CFI	373G>A	**Gly125Arg**	NA	2266	1400	0.0221	0.0000		T	D	NA	None	NA	NA
23	4	110685802	CFI	373G>A	**Gly125Arg**	NA	2335	797	0.0214	0.0000		T	D	NA	None	NA	NA
6	4	110685820	CFI	355G>A	**Gly119Arg**	rs141853578	17823	16127	0.2900	0.0800		T	D	0.0529	Table 3	3.64	6.27E-10
1	4	110685820	CFI	355G>A	**Gly119Arg**	rs141853578	521	627	0.6718	0.0797		T	D	0.0529	Table 3	3.64	6.27E-10
7	4	110685820	CFI	355G>A	**Gly119Arg**	rs141853578	1831	1367	0.4096	0.0366		T	D	0.0529	Table 3	3.64	6.27E-10
10	4	110685820	CFI	355G>A	**Gly119Arg**	rs141853578	2266	1400	0.2207	0.0714		T	D	0.0529	Table 3	3.64	6.27E-10
16	4	110685820	CFI	355G>A	**Gly119Arg**	rs141853578	1589	1386	0.4091	0.0361	1 family 4 carriers	T	D	0.0529	Table 3	3.64	6.27E-10
17	4	110685820	CFI	355G>A	**Gly119Arg**	rs141853578	1712	781	0.2044	0.0640		T	D	0.0529	Table 3	3.64	6.27E-10
20	4	110685820	CFI	355G>A	**Gly119Arg**	rs141853578	3567	3937	0.2803	0.0127		T	D	0.0529	Table 3	3.64	6.27E-10
23	4	110685820	CFI	355G>A	**Gly119Arg**	rs141853578	2335	797	0.2784	0.0000		T	D	0.0529	Table 3	3.64	6.27E-10
10	4	110687712	CFI	326A>C	**Glu109Ala**	NA	2266	1400	0.0221	0.0000		T	B	NA	None	NA	NA

Source	Chr	Position	Gene	cDNA change (c.)	Protein change (p.)	rs-number	Number Cases	Number Controls	MAF Cases (%)	MAF Controls (%)	Families (incl proband)	SIFT	Poly Phen	ExAC (%)	Function	IAMDGC Odds Ratio	IAMDGC P-Value
17	4	110687712	CFI	326A>C	**Glu109Ala**	NA	1712	781	0.0292	0.0000		T	B	NA	None	NA	NA
23	4	110687719	CFI	319A>G	Thr107Ala	rs201419000	2335	797	0.0000	0.0627		T	B	0.0420	None	NA	NA
23	4	110687722	CFI	316T>C	Cys106Arg	rs775074891	2335	797	0.0214	0.0000		D	D	0.0008	None	NA	NA
23	4	110687793	CFI	245T>C	Phe82Ser	NA	2335	797	0.0214	0.0000		T	D	NA	None	NA	NA
23	4	110687829	CFI	209A>C	Asn70Thr	rs749553820	2335	797	0.0214	0.1255		T	B	0.0049	None	NA	NA
10	4	110687847	CFI	191C>T	**Pro64Leu**	rs773187287	2266	1400	0.0221	0.0000		T	D	0.0190	None	NA	NA
17	4	110687847	CFI	191C>T	**Pro64Leu**	rs773187287	1712	781	0.0292	0.0000		T	D	0.0190	None	NA	NA
23	4	110687875	CFI	163A>T	Ile55Phe	NA	2335	797	0.0214	0.0000		D	D	NA	None	NA	NA
10	4	110687876	CFI	162C>A	Cys54Ter	NA	2266	1400	0.0000	0.0357		NA	NA	NA	None	NA	NA
10	4	110687878	CFI	160T>C	**Cys54Arg**	NA	2266	1400	0.0221	0.0000		D	D	NA	None	NA	NA
23	4	110687878	CFI	160T>C	**Cys54Arg**	NA	2335	797	0.0214	0.0000		D	D	NA	None	NA	NA
10	4	110687890	CFI	148C>G	**Pro50Ala**	rs144082872	2266	1400	0.0441	0.0000		T	D	0.0107	Table 3	NA	NA
17	4	110687890	CFI	148C>G	**Pro50Ala**	rs144082872	1712	781	0.0584	0.0000		T	D	0.0107	Table 3	NA	NA
23	4	110687890	CFI	148C>G	**Pro50Ala**	rs144082872	2335	797	0.0214	0.0000		T	D	0.0107	Table 3	NA	NA
10	4	110687908	CFI	130G>A	**Asp44Asn**	rs374036714	2266	1400	0.0441	0.0000		T	B	0.0016	None	NA	NA
17	4	110687908	CFI	130G>A	**Asp44Asn**	rs374036714	1712	781	0.0292	0.0000		T	B	0.0016	None	NA	NA
23	4	110687908	CFI	130G>A	**Asp44Asn**	rs374036714	2335	797	0.0214	0.0000		T	B	0.0016	None	NA	NA
10	4	110687919	CFI	119A>G	**His40Arg**	rs767734237	2266	1400	0.0221	0.0000		T	B	0.0016	None	NA	NA
17	4	110687919	CFI	119A>G	**His40Arg**	rs767734237	1712	781	0.0292	0.0000		T	B	0.0016	None	NA	NA
23	4	110687919	CFI	119A>G	**His40Arg**	rs767734237	2335	797	0.0214	0.0000		T	B	0.0016	None	NA	NA
10	4	110687980	CFI	58A>G	Val20Ile	NA	2266	1400	0.0000	0.0357		T	B	NA	None	NA	NA
6	5	39285311	C9	1670A>G	Asn557Ser	rs138480043	17829	16142	0.0100	0.0100		T	B	0.0264	None	1.26	0.81
6	5	39288885	C9	1585G>A	Ala529Thr	rs137891079	17829	16144	0.0500	0.0800		T	B	0.0512	None	0.67	0.22
6	5	39289043	C9	1427T>C	Ile476Thr	rs141645272	17829	16141	0.2300	0.1900		D	D	0.1131	None	1.24	0.23
6	5	39306855	C9	1280C>G	Ser427Ter	rs121909594	17822	16131	0.0100	0.0000		NA	NA	0.0008	None	2.51	0.35
6	5	39306856	C9	1279T>A	Ser427Thr	rs34421659	17832	16144	0.0000	0.0000		T	B	0.3338	None	2.89	0.51
6	5	39311336	C9	1014T>A	Phe338Leu	rs141600725	17806	16104	0.2200	0.2100		T	D	0.0849	None	1.04	0.84
6	5	39311416	C9	934G>A	Asp312Asn	rs147710831	17782	16105	0.0400	0.0300		T	D	0.0231	None	1.24	0.61
6	5	39311463	C9	887del	His296Leufs*2	NA	17828	16142	0.0000	0.0000		NA	NA	NA	None	1.08	0.95
6	5	39315912	C9	835A>G	Thr279Ala	rs183125896	17786	16106	0.0000	0.0100		T	B	0.1001	None	0.47	0.44

6	5	39331786	C9	607A>G	**Ile203Val**	rs13361416	17832	16144	0.1100	0.1400		T	B	1.0280	None	0.73	0.17
17	5	39331786	C9	607A>G	**Ile203Val**	rs13361416	1676	745	0.1492	0.2013		T	B	1.0280	None	0.73	0.17
6	5	39331813	C9	580C>T	Arg194Ter	rs146217095	17832	16141	0.0000	0.0000		NA	NA	0.0107	None	3.14	0.48
6	5	39331894	C9	499C>T	**Pro167Ser**	rs34882957	17832	16144	1.5600	0.8700		D	D	0.4711	Table 3	1.80	1.62E-14
7	5	39331894	C9	499C>T	**Pro167Ser**	rs34882957	1831	1367	1.6658	1.1339	1 family with 4 carriers	D	D	0.4711	Table 3	1.80	1.62E-14
16	5	39331894	C9	499C>T	**Pro167Ser**	rs34882957	1589	1386	1.5419	1.0101	2 families with 5 carriers	D	D	0.4711	Table 3	1.80	1.62E-14
17	5	39331894	C9	499C>T	**Pro167Ser**	rs34882957	1676	745	1.5811	0.7383		D	D	0.4711	Table 3	1.80	1.62E-14
6	5	39341345	C9	379G>T	Asp127Tyr	rs696763	17832	16144	0.0100	0.0000		D	D	0.3039	None	2.69	0.34
6	5	39341348	C9	376G>A	Gly126Arg	rs199939436	17826	16142	0.0100	0.0300		D	D	0.0330	None	0.43	0.13
6	5	39341369	C9	355T>G	Cys119Gly	rs121909593	17832	16144	0.0400	0.0400		D	D	0.0331	None	1.23	0.61
6	5	39341372	C9	352C>T	**Arg118Trp**	rs147701327	17829	16141	0.0400	0.0200		D	P	0.0190	Table 3	1.85	0.18
7	5	39341372	C9	352C>T	**Arg118Trp**	rs147701327	1831	1367	0.0819	0.0732	1 family with 3 carriers	D	P	0.0190	Table 3	1.85	0.18
12#	5	39341378	C9	346C>T	Arg116Ter	rs121909592	198	396	1.0101	3.1566		NA	NA	0.0827	Table 3	NA	NA
6	5	39341682	C9	304G>A	Gly102Arg	rs145819975	17827	16139	0.0000	0.0000		D	D	0.0091	None	3.25	0.46
6	5	39341730	C9	256C>T	Arg86Ter	rs148881448	17826	16139	0.0200	0.0100		NA	NA	0.0091	None	1.79	0.38
6	5	39342243	C9	133A>T	**Met45Leu**	rs41271047	17828	16139	0.3300	0.3500		T	B	0.2060	None	0.93	0.62
17	5	39342243	C9	133A>T	**Met45Leu**	rs41271047	1676	745	0.3282	0.3356		T	B	0.2060	None	0.93	0.62
6	19	6677963	C3	4922A>G	**Glu1641Gly**	rs34370481	17832	16144	0.0100	0.0100		D	P	0.0033	None	0.74	0.68
23	19	6677963	C3	4922A>G	**Glu1641Gly**	rs34370481	2335	797	0.0000	0.0627		D	P	0.0033	None	0.74	0.68
23	19	6678018	C3	4867G>C	Gly1623Arg	NA	2335	797	0.0214	0.0000		D	D	NA	None	NA	NA
6	19	6678030	C3	4855A>C	**Ser1619Arg**	rs2230210	17832	16144	0.1200	0.1400		T	P	0.1096	None	0.90	0.71
3	19	6678030	C3	4855A>C	**Ser1619Arg**	rs2230210	1553	2523	0.3863	0.2576		T	P	0.1096	None	0.90	0.71
17	19	6678030	C3	4855A>C	**Ser1619Arg**	rs2230210	1676	745	0.1492	0.3356		T	P	0.1096	None	0.90	0.71
23	19	6678030	C3	4855A>C	**Ser1619Arg**	rs2230210	2335	797	0.1285	0.2509		T	P	0.1096	None	0.90	0.71
6	19	6678430	C3	4667A>G	**Asn1556Ser**	rs139381845	17832	16144	0.0100	0.0200		T	B	0.0033	None	0.74	0.65
23	19	6678430	C3	4667A>G	**Asn1556Ser**	rs139381845	2335	797	0.0428	0.0000		T	B	0.0033	None	0.74	0.65
23	19	6678433	C3	4664C>G	Ser1555Cys	rs750559710	2335	797	0.0214	0.0000		D	D	NA	None	0.36	0.53
6	19	6678452	C3	4645C>A	Leu1549Met	rs149202905	17832	16144	0.0000	0.0000		T	P	0.1164	Table 3	1.17	0.90

Source	Chr	Position	Gene	cDNA change (c.)	Protein change (p.)	rs-number	Number Cases	Number Controls	MAF Cases (%)	MAF Controls (%)	Families (incl proband)	SIFT	Poly Phen	ExAC (%)	Function	IAMDGC Odds Ratio	IAMDGC P-Value
23	19	6678454	C3	4643G>T	Arg1548Leu	NA	2335	797	0.0214	0.0000		D	B	NA	None	NA	NA
6	19	6679172	C3	4594C>T	**Arg1532Trp**	rs199911426	17831	16142	0.0100	0.0000		D	D	0.0066	None	12.29	0.04
23	19	6679172	C3	4594C>T	**Arg1532Trp**	rs199911426	2335	797	0.0214	0.0000		D	D	0.0066	None	12.29	0.04
6	19	6679429	C3	4535G>A	**Arg1512His**	rs142868256	17832	16144	0.0000	0.0100		D	P	0.0140	None	0.48	0.47
23	19	6679429	C3	4535G>A	**Arg1512His**	rs142868256	2335	797	0.0214	0.0000		D	P	0.0140	None	0.48	0.47
6	19	6679493	C3	4471C>T	**Arg1491Trp**	rs140928439	17830	16142	0.0100	0.0200		D	D	0.0132	None	0.59	0.44
23	19	6679493	C3	4471C>T	**Arg1491Trp**	rs140928439	2335	797	0.0000	0.0627		D	D	0.0132	None	0.59	0.44
23	19	6680184	C3	4441G>A	Ala1481Thr	NA	2335	797	0.0214	0.0000		T	P	NA	None	NA	NA
6	19	6680256	C3	4369G>C	Asp1457His	rs113742728	17832	16144	0.0000	0.0000		T	D	0.0363	None	2.81	0.52
6	19	6680261	C3	4364A>G	**Glu1455Gly**	NA	17832	16144	0.0000	0.0000		T	P	0.0033	None	0.30	0.45
23	19	6680261	C3	4364A>G	**Glu1455Gly**	rs761663480	2335	797	0.0214	0.0000		T	P	0.0033	None	0.30	0.45
6	19	6681983	C3	4319A>C	**Asp1440Ala**	rs147116781	17832	16142	0.0200	0.0200		D	B	0.0297	None	0.85	0.78
23	19	6681983	C3	4319A>C	**Asp1440Ala**	rs147116781	2335	797	0.0214	0.0627		D	B	0.0297	None	0.85	0.78
6	19	6682236	C3	4177C>T	**Arg1393Trp**	rs148227405	17831	16144	0.0100	0.0100		D	D	0.0041	None	0.87	0.88
23	19	6682236	C3	4177C>T	**Arg1393Trp**	rs148227405	2335	797	0.0214	0.0627		D	D	0.0041	None	0.87	0.88
6	19	6684423	C3	4148C>A	Thr1383Asn	rs139100972	17827	16139	0.0200	0.0100		T	B	0.0091	None	2.73	0.18
23	19	6684591	C3	4100T>C	Ile1367Thr	rs11569541	2335	797	0.0428	0.0000		D	B	0.0783	None	NA	NA
6	19	6684607	C3	4084G>A	**Asp1362Asn**	NA	17832	16144	0.0100	0.0100		T	B	0.0049	None	1.29	0.73
23	19	6684607	C3	4084G>A	**Asp1362Asn**	rs368788004	2335	797	0.0214	0.0627		T	B	0.0049	None	1.29	0.73
23	19	6685109	C3	3859C>T	Pro1287Ser	NA	2335	797	0.0214	0.0000		T	D	NA	None	NA	NA
6	19	6685157	C3	3811G>T	**Ala1271Ser**	NA	17832	16144	0.0000	0.0000		T	D	NA	None	3.28	0.46
23	19	6685157	C3	3811G>T	**Ala1271Ser**	NA	2335	797	0.0214	0.0000		T	D	NA	None	3.28	0.46
23	19	6686185	C3	3760C>T	Arg1254Cys	NA	2335	797	0.0000	0.0627		D	D	NA	None	0.35	0.51
6	19	6686257	C3	3688G>A	**Val1230Met**	rs147113695	17832	16144	0.0000	0.0000		D	D	0.0025	None	3.49	0.44
23	19	6686257	C3	3688G>A	**Val1230Met**	rs147113695	2335	797	0.0000	0.0627		D	D	0.0025	None	3.49	0.44
6	19	6686274	C3	3671G>A	**Gly1224Asp**	rs11569534	17832	16144	0.0100	0.0100		T	B	0.1837	None	0.47	0.34
23	19	6686274	C3	3671G>A	**Gly1224Asp**	rs11569534	2335	797	0.0857	0.0000		T	B	0.1837	None	0.47	0.34
6	19	6686290	C3	3655C>T	**Arg1219Cys**	NA	17832	16144	0.0100	0.0100		T	D	0.0041	None	1.05	0.95
23	19	6686290	C3	3655C>T	**Arg1219Cys**	rs373102036	2335	797	0.0214	0.0000		T	D	0.0041	None	1.05	0.95
23	19	6686298	C3	3647A>T	Asp1216Val	NA	2335	797	0.0214	0.0000		D	D	NA	None	NA	NA

23	19	6686901 C3	3502A>G	Ser1168Gly	rs746343439	2335	797	0.0214	0.0000	D	P	0.0008	None	NA	NA
6	19	6690654 C3	3475G>A	**Glu1159Lys**	NA	17832	16144	0.0000	0.0000	T	B	0.0008	None	0.38	0.55
23	19	6690654 C3	3475G>A	**Glu1159Lys**	rs367654112	2335	797	0.0214	0.0000	T	B	0.0008	None	0.38	0.55
6	19	6690698 C3	3431C>T	**Thr1144Met**	rs150237828	17832	16144	0.0200	0.0100	D	P	0.0041	None	2.41	0.26
23	19	6690698 C3	3431C>T	**Thr1144Met**	rs150237828	2335	797	0.0214	0.0000	D	P	0.0041	None	2.41	0.26
6	19	6690729 C3	3400C>T	Arg1134Trp	rs138900723	17824	16133	0.0100	0.0100	D	D	0.0033	None	1.33	0.71
23	19	6693020 C3	3305G>A	Gly1102Glu	NA	2335	797	0.0214	0.0000	D	D	NA	None	NA	NA
6	19	6693026 C3	3299T>C	**Leu1100Pro**	NA	16364	15422	0.0000	0.0100	D	D	0.0008	None	0.36	0.27
23	19	6693026 C3	3299T>C	**Leu1100Pro**	rs750654763	2335	797	0.0214	0.0000	D	D	0.0008	None	0.36	0.27
6	19	6693470 C3	3183A>T	**Gln1061His**	NA	17832	16144	0.0100	0.0000	T	B	0.0197	None	1.81	0.58
23	19	6693470 C3	3183A>T	**Gln1061His**	rs373054812	2335	797	0.0214	0.0000	T	B	0.0197	None	1.81	0.58
6	19	6693473 C3	3180A>T	**Arg1060Ser**	rs142393845	17831	16142	0.0000	0.0000	D	D	0.0010	None	1.07	0.96
23	19	6693473 C3	3180A>T	**Arg1060Ser**	rs142393845	2335	797	0.0000	0.0627	D	D	0.0010	None	1.07	0.96
23	19	6694484 C3	3112G>A	Gly1038Ser	NA	2335	797	0.0214	0.0000	T	D	NA	None	NA	NA
23	19	6694547 C3	3049G>C	Gly1017Arg	rs757640085	2335	797	0.0214	0.0000	T	P	0.0008	None	NA	NA
6	19	6694573 C3	3023C>T	**Ser1008Leu**	NA	17832	16144	0.0100	0.0000	T	D	0.0041	None	3.27	0.20
23	19	6694573 C3	3023C>T	**Ser1008Leu**	rs746172422	2335	797	0.0214	0.0000	T	D	0.0041	None	3.27	0.20
23	19	6697518 C3	2633C>A	Thr878Asn	NA	2335	797	0.0000	0.0627	T	B	NA	None	0.35	0.52
23	19	6697542 C3	2609C>T	Pro870Leu	NA	2335	797	0.0000	0.0627	D	D	NA	None	NA	NA
6	19	6697682 C3	2564G>A	Arg855Gln	rs199784156	17832	16144	0.0100	0.0200	T	B	0.0058	None	0.36	0.15
6	19	6702552 C3	2284G>A	Val762Ile	NA	17831	16143	0.0000	0.0000	T	B	0.0016	None	3.33	0.45
6	19	6707125 C3	2207G>A	**Arg736Gln**	NA	17831	16141	0.0100	0.0200	T	B	0.0052	None	0.60	0.47
23	19	6707125 C3	2207G>A	**Arg736Gln**	rs578116271	2335	797	0.0214	0.0000	T	B	0.0052	None	0.60	0.47
6	19	6707129 C3	2203C>T	**Arg735Trp**	rs117793540	17831	16141	0.2800	0.2800	D	D	0.2086	Table 3	0.99	0.95
3	19	6707129 C3	2203C>T	**Arg735Trp**	rs117793540	1504	2294	0.3657	0.0654	D	D	0.2086	Table 3	0.99	0.95
17	19	6707129 C3	2203C>T	**Arg735Trp**	rs117793540	1676	745	0.2387	0.2685	D	D	0.2086	Table 3	0.99	0.95
23	19	6707129 C3	2203C>T	**Arg735Trp**	rs117793540	2335	797	0.3854	0.1882	D	D	0.2086	Table 3	0.99	0.95
23	19	6707206 C3	2126G>A	Arg709His	NA	2335	797	0.0214	0.0000	D	B	NA	None	3.16	0.48
23	19	6707225 C3	2107A>T	Met703Leu	NA	2335	797	0.0000	0.0627	T	D	NA	None	0.35	0.52
6	19	6707228 C3	2104C>T	**Pro702Ser**	NA	17828	16137	0.0000	0.0000	T	P	0.0009	None	3.24	0.47
23	19	6707228 C3	2104C>T	**Pro702Ser**	rs753504699	2335	797	0.0214	0.0000	T	P	0.0009	None	3.24	0.47
6	19	6707536 C3	1988C>G	Pro663Arg	rs112187261	17830	16141	0.0100	0.0100	D	P	0.0025	None	1.07	0.92
23	19	6707877 C3	1909G>C	Gly637Arg	rs149850773	2335	797	0.0214	0.0627	D	D	0.0215	None	NA	NA

Source	Chr	Position	Gene	cDNA change (c.)	Protein change (p.)	rs-number	Number Cases	Number Controls	MAF Cases (%)	MAF Controls (%)	Families (incl proband)	SIFT	Poly Phen	ExAC (%)	Function	IAMDGC Odds Ratio	IAMDGC P-Value
6	19	6707888	C3	1898A>G	**Lys633Arg**	rs140655115	17830	16140	0.0800	0.0800		T	B	0.0398	Table 3	1.09	0.77
17	19	6707888	C3	1898A>G	**Lys633Arg**	rs140655115	1676	745	0.0895	0.2013		T	B	0.0398	Table 3	1.09	0.77
23	19	6707888	C3	1898A>G	**Lys633Arg**	rs140655115	2335	797	0.0642	0.0000		T	B	0.0398	Table 3	1.09	0.77
6	19	6707913	C3	1873A>T	Ile625Phe	rs144432231	17805	16119	0.0200	0.0300		T	B	0.0465	None	0.67	0.44
6	19	6707931	C3	1855G>A	**Val619Met**	rs146613648	17832	16139	0.1500	0.0600		T	P	0.0291	None	2.66	2.38E-04
17	19	6707931	C3	1855G>A	**Val619Met**	rs146613648	1676	745	0.1193	0.1342		T	P	0.0291	None	2.66	2.38E-04
23	19	6707931	C3	1855G>A	**Val619Met**	rs146613648	2335	797	0.0642	0.2509		T	P	0.0291	None	2.66	2.38E-04
6	19	6709721	C3	1819A>G	**Lys607Glu**	rs140637006	16350	15406	0.0100	0.0100		D	P	0.0025	None	0.75	0.74
23	19	6709721	C3	1819A>G	**Lys607Glu**	rs140637006	2335	797	0.0214	0.0000		D	P	0.0025	None	0.75	0.74
23	19	6709798	C3	1742T>C	Met581Thr	NA	2335	797	0.0214	0.0000		T	B	NA	None	NA	NA
23	19	6709838	C3	1702G>A	Gly568Ser	rs749836289	2335	797	0.0214	0.0000		T	B	0.0008	None	NA	NA
6	19	6710712	C3	1624G>A	Gly542Ser	rs375626292	17829	16138	0.0000	0.0000		T	P	0.0017	None	3.17	0.47
6	19	6710718	C3	1618G>T	Ala540Ser	rs201237210	17827	16138	0.0100	0.0100		T	B	0.0059	None	0.66	0.61
6	19	6711066	C3	1411C>T	**Leu471Phe**	NA	17832	16144	0.0000	0.0000		D	D	NA	None	3.13	0.48
23	19	6711066	C3	1411C>T	**Leu471Phe**	NA	2335	797	0.0214	0.0000		D	D	NA	None	3.13	0.48
6	19	6711070	C3	1407G>C	**Glu469Asp**	rs11569422	17832	16144	0.0100	0.0200		T	B	0.3937	None	0.90	0.87
23	19	6711070	C3	1407G>C	**Glu469Asp**	rs11569422	2335	797	0.1285	0.0627		T	B	0.3937	None	0.90	0.87
23	19	6711204	C3	1273C>T	Arg425Cys	rs200967589	2335	797	0.0214	0.0627		D	D	0.0174	None	NA	NA
23	19	6712275	C3	1262G>A	Ser421Asn	NA	2335	797	0.0214	0.0000		T	B	NA	None	NA	NA
6	19	6712596	C3	1042A>G	Ile348Val	rs141737564	17821	16124	0.0000	0.0100		D	P	0.0049	None	0.37	0.28
23	19	6713212	C3	991A>C	Ile331Leu	NA	2335	797	0.0214	0.0000		T	B	NA	None	0.36	0.53
6	19	6713275	C3	928G>A	Gly310Arg	rs139527231	16365	15423	0.0000	0.0100		T	D	0.0050	None	0.15	0.15
23	19	6713313	C3	890C>T	Ser297Leu	rs753979097	2335	797	0.0214	0.0000		T	B	0.0017	None	NA	NA
23	19	6714007	C3	769G>A	Ala257Thr	rs200918899	2335	797	0.0214	0.0000		D	D	0.0050	None	NA	NA
6	19	6714079	C3	697G>A	Glu233Lys	rs373896614	16365	15423	0.0100	0.0100		D	D	0.0017	None	0.76	0.75
6	19	6714400	C3	562G>A	Val188Ile	NA	16365	15423	0.0200	0.0000		T	B	0.0050	None	3.84	0.13
23	19	6714400	C3	562G>A	Val188Ile	rs765821415	2335	797	0.0642	0.0000		T	B	0.0050	None	3.84	0.13
3	19	6718128	C3	481C>T	**Arg161Trp**	rs776423109	964	1625	0.1556	0.0308		D	D	NA	Table 3	NA	NA

7	19	6718128	C3	481C>T	**Arg161Trp**	rs776423109	1831	1367	0.1092	0.0000	2 families with 9 carriers	D	D	NA	Table 3	NA	NA
6	19	6718146	C3	463A>C	**Lys155Gln**	rs147859257	17830	16143	1.2400	0.4300		T	B	0.3362	Table 3	2.87	3.08E-28
7	19	6718146	C3	463A>C	**Lys155Gln**	rs147859257	1831	1367	1.1469	0.5852	1 family with 2 carriers	T	B	0.3362	Table 3	2.87	3.08E-28
8	19	6718146	C3	463A>C	**Lys155Gln**	rs147859257	3961	54412	1.4769	0.5394		T	B	0.3362	Table 3	2.87	3.08E-28
16	19	6718146	C3	463A>C	**Lys155Gln**	rs147859257	1589	1386	0.9125	0.5051	3 families with 9 carriers	T	B	0.3362	Table 3	2.87	3.08E-28
17	19	6718146	C3	463A>C	**Lys155Gln**	rs147859257	1676	745	1.1933	0.0671		T	B	0.3362	Table 3	2.87	3.08E-28
23	19	6718146	C3	463A>C	**Lys155Gln**	rs147859257	2335	797	1.0278	0.3137		T	B	0.3362	Table 3	2.87	3.08E-28
3	19	6719296	C3	193A>C	**Lys65Gln**	rs539992721	Unknown	Unknown	0.1400	0.0000		T	D	0.0058	Table 3	NA	NA
23	19	6719296	C3	193A>C	**Lys65Gln**	rs539992721	2335	797	0.0642	0.0000		T	D	0.0058	Table 3	NA	NA
6	19	6719308	C3	181G>A	**Asp61Asn**	NA	17832	16144	0.0100	0.0000		T	D	0.0016	None	5.30	0.24
23	19	6719308	C3	181G>A	**Asp61Asn**	rs778521833	2335	797	0.0214	0.0000		T	D	0.0016	None	5.30	0.24
23	19	6720575	C3	26T>C	Leu9Pro	rs138214338	2335	797	0.1285	0.1255		D	B	0.0547	None	NA	NA

Bold: found in multiple AMD studies. # In recent genome builds the Arg95Ter nonsense variant is notated as Arg116Ter. Source: 1 Alexander2014; 2 Boon2008; 3 Duvvari2014; 4 Duvvari2015; 5 Duvvari2016; 6 Fritsche2016; 7 Geerlings2016; 8 Helgason2013; 9 Hoffman2014; 10 Kavanagh2015; 11 Miyake2015; 12 Nishiguchi2012#; 13 Pras2015; 14 Raychaudhuri2011; 15 Recalde2016; 16 Saksens2016; 17 Seddon2013; 18 Triebwasser2015; 19 VandeVen2012; 20 VandeVen2013; 21 Wagner2016; 22 Yu2015; 23 Zhan2013

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Nicole T.M. Saksens*
Maartje J. Geerlings*
Bjorn Bakker
Tina Schick
Mohamed R. Daha
Sascha Fauser
Camiel J.F. Boon
Eiko K. de Jong
Carel B. Hoyng
Anneke I. den Hollander

*these authors contributed equally to this study

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RARE GENETIC VARIANTS ASSOCIATED WITH DEVELOPMENT OF AGE-RELATED MACULAR DEGENERATION

ABSTRACT

Importance: Rare variants in the complement genes *CFH, CFI, C9* and *C3* were found to be highly associated with age-related macular degeneration (AMD); however, the effect on clinical characteristics and familial segregation by these variants is lacking.

Objectives: To determine the contribution of rare *CFH* p.Arg1210Cys, *CFI* p.Gly119Arg, *C9* p.Pro167Ser, and *C3* p.Lys155Gln variants in the development of AMD in 22 multiplex families and to describe clinical differences in carriers versus non-carriers in these families and a large case-control cohort.

Design, setting and participants: This retrospective case-control study included 114 affected and 60 unaffected members of 22 multiplex families with AMD as well as 1589 unrelated patients with AMD and 1386 unrelated control individuals enrolled in the European Genetic Database (EUGENDA). Patients were recruited from March 29, 2006, to April 26, 2013, and data were collected from April 20, 2012, to May 7, 2014. All participants underwent an extensive ophthalmic examination and completed a questionnaire. Venous blood samples were obtained from all participants for genetic analysis, including whole-exome sequencing and measurements of complement activation. Data were analyzed from September 23, 2014, to November 4, 2015.

Main Outcome Measures: Differences between carriers and noncarriers of rare variants in age at onset of symptoms, the family history of AMD, complement activation levels (C3d/C3 ratio), the presence of reticular pseudodrusen, and AMD phenotype.

Results: Among the 114 affected and 60 unaffected members of 22 multiplex families with AMD and the 1598 unrelated patients with AMD and 1386 controls in the EUGENDA cohort who underwent analysis, the presence of the *CFI* p.Gly119Arg, *C9* p.Pro167Ser, or *C3* p.Lys155Gln variant was confirmed in 18 individuals in 5 families but did not completely segregate with the disease. In the case-control cohort, the 91 affected carriers of these variants were younger at symptom onset (mean [SD] age, 67.4 [8.5] versus 71.3 [8.9] years; P = .01) and more often reported a positive family history (35 of 79 [44.3%] versus 367 of 1201 [30.6%]; P = .008) compared with the 1498 noncarriers. Patients with advanced atrophic AMD carried these rare variants more frequently than patients with neovascular AMD (11 of 93 [11.8%] versus 40 of 835 [4.8%]; P = .04).

Conclusions and relevance: Previously reported rare variants do not completely segregate within families with AMD. However, patients carrying these rare variants differ clinically from non-carriers by an earlier age at symptom onset, higher prevalence of a positive family history, and by AMD phenotype. These results suggest that genetic tests for AMD might be designed to detect common and rare genetic variants, especially in families, because rare variants contribute to the age at onset and progression of the disease.

INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of irreversible, central visual loss in the elderly population in developed countries.[1] A combination of genetic and non-genetic factors plays a role in the development and progression of this multifactorial disease.[2,3] Genome-wide association studies have identified common genetic risk variants that are strongly associated with AMD, such the p.Tyr402His (rs1061170) variant in the complement factor H (*CFH*) gene (HGNC 4483), and the p.Ala69Ser (rs10490924) variant in the age-related maculopathy susceptibility 2 *(ARMS2)* gene (HGNC 32685).[2,4,5]

Previous family- and twin-studies have demonstrated a strong genetic component and aggregation of AMD within families.[6-9] Approximately 20-30% of the patients have a positive family history for AMD,[7,10-12] which has been reported as a significant risk factor for AMD. A positive family history also has been associated with an earlier age at onset of disease.[13-17] Clustering of known common genetic risk factors does not fully explain the number of affected family members in large, densely affected families.[7] Several recent studies have identified rare genetic variants, that strongly increase the risk for AMD, including *CFH* p.Arg1210Cys, *CFI* (HGNC 5394) p.Gly119Arg, *C9* p.Pro167Ser (HGNC 1358), and *C3* (HGNC 1318) p.Lys155Gln.[15,18-21] These rare variants are located in genes of the complement system, which plays a major role in the pathogenesis of AMD.[2,22] Owing to their strong effect size, these rare, highly penetrant genetic variants may account for clustering of AMD in families and lead to more severe disease. Highly penetrant variants have been identified in families with AMD, thus confirming the hypothesis that rare variants cluster in families.[15-17] Understanding the contributions of these rare variants to the clinical characteristics of AMD is important because carriying these variants may have diagnostic, predictive and therapeutic consequences for carriers.

The aim of the present study was to determine the contribution of known rare genetic variants in the development of AMD in large, multiplex families with AMD. In addition, we aimed to describe differences in clinical characteristics in carriers compared with non-carriers of these rare genetic variants, in families and a large case-control cohort.

METHODS

Participants

In this retrospective study, we evaluated 114 affected and 60 unaffected members of 22 multiplex families with AMD. In addition, we analyzed a case-control cohort of 1589 unrelated patients with AMD and 1386 unrelated control individuals from the European Genetic Database (EUGENDA). This study was approved by the local ethics committees of Commissie Mensgebonden Onderzoek Regio Arnhem-Nijmegen and University Hospital Cologne on research involving human participants, and met the criteria of the Declaration of Helsinki.[23] Before enrollment in EUGENDA, all participants provided written informed consent and completed a detailed questionnaire on their medical history, age at onset of first symptoms, family history of AMD, and lifestyle factors. For the case-control cohort, a family history positive for AMD was defined as at least two first-degree relatives (parents and/or siblings) with AMD or possible AMD.

Patients were recruited from March 29, 2006, to April 26, 2013, and data were collected from April 20, 2012, to May 7, 2014. Each participant of the EUGENDA cohort, and all members of the 22 families, underwent digital color fundus photography and spectral-domain optical coherence tomography (OCT, Spectralis; Heidelberg Engineering, Heidelberg, Germany) after pupillary dilation. Digital non-stereoscopic 30° color fundus photography centered on the fovea was performed (TRC 50IX camera; Topcon Corporation, Tokyo, Japan). Spectral-domain OCT volume scans consisted of 19 or 37 parallel OCT B-scans for analysis, which covered a macular area of 6x4 mm. For each OCT B-scan, 20 images were averaged using the automated real-time function.[24]_ENREF_21 Color fundus photographs and OCT scans of both eyes of all individuals were evaluated by two independent certified reading center graders, including one of us (T.S.), according to the standard protocol of the Cologne Image Reading Center and Laboratory (CIRCL).[24] We classified AMD by the presence of pigmentary changes with at least 10 small drusen (diameter, <63 μm) or the presence of intermediate (diameter, 63-124 μm) or large (diameter, ≥125 μm) drusen in the Early Treatment Diabetic Retinopathy Study grid. Advanced AMD was defined as AMD with subfoveal geographic atrophy (GA) or choroidal neovascularization (CNV) in at least one eye. Age at onset of AMD was defined as the age at which the first visual symptoms occurred. Controls were classified as having no abnormalities or only small drusen or pigmentary abnormalities, and were 60 years or older. In addition, in 479 individuals, infrared images and spectral domain OCT images were evaluated for the presence of reticular pseudodrusen by one of us (TS).

Genotyping

Whole exome sequencing was used to genotype 85 affected members of 22 multiplex families with AMD. The samples were sequenced at the Erasmus Medical Center using DNA obtained from venous blood after extraction using standard procedures. The DNA was fragmented using shearing according to the manufacturer's instructions (Adaptive Focused Acoustics; Covaris, Inc., Woburn, MA), and a DNA library preparation kit (Kapa Biosystems, Inc., Wilmington, MA) was used on sequencer workstation (Sciclone NGs; Caliper Life Sciences, Hopkinton, MA). Exome capture was achieved using an exome solution kit (Nimblegen SeqCap EZ V2; Roche Nimblegen, Inc., Madison, WI), designed to capture more than 44 Mb of exonic regions. Paired-end 2 ×100 sequencing was performed on a device (HiSeq2000; Illumina, Inc) using a reagent kit (TruSeq V3; Illumina, Inc.). Downstream analyses included demultiplexing (CASAVA software, Illumina) and alignment to the hg19 reference genome (Genome Reference Consortium Human Reference[25] [http://www.ncbi.nlm.nih.gov/assembly/GCF_000001405.25/]) by Burrows-Wheeler alignment tool.[26] Alignments were sorted by Picard (http://broadinstitute.github.io/picard) and subsequently processed using the Genome Analysis Toolkit (GATK)(indel realignment and Base-Quality Score Recalibration).[27] Finally, polymerase chain reaction duplicates were marked by Picard, mean depth of coverage was determined using GATK, and Freemix values were estimated through verifyBAMid.[28] Samples that passed technical quality control metrics were genotyped to genomic variant format level through the GATK haplotype caller. Insertions, deletions, and single nucleotide variants were filtered separately using the GATK Variant-Quality Score Recalibration, and annotated using the ANNOVAR tool.[29]

We used filtering steps to select the previously associated variants in the *CFH* (p.Arg1210Cys; rs121913059), *CFI* (p.Gly119Arg; rs141853578), *C9* (p.Pro167Ser; rs34882957) genes and *C3* (Lys155Gln; rs147859257) from the exome files of the 85 affected family members. The annotation of the identified variants was confirmed by Sanger sequencing using primers designed with Primer3 software (http://primer3.ut.ee) (**eTable 1**). The variants were also analyzed in the patients of the index families of whom exome sequencing was not available (n=15) using Sanger sequencing.

Genotyping of the rare *CFH* p.Arg1210Cys, *CFI* p.Gly119Arg, *C9* p.Pro167Ser, and *C3* p.Lys155Gln genetic variants was performed in all 2975 included participants of the EUGENDA case-control cohort. Genotyping of the *CFI* p.Gly119Arg variant was performed using a custom-made assay (TaqMan; Life Technologies), as described previously.[18] Genotyping of the *CFH* p.Arg1210Cys, *C9* p.Pro167Ser and *C3* p.Lys155Gln variants was performed by competitive, allele-specific, polymerase chain reaction assays (KASP SNP Genotyping System; LGC Group) for *CFH* as previously described[30] and *C9*, *C3*, according to the manufacturers' recommendations (**eTable 2**).

Complement measurements

Levels of complement component C3 and the activation fragment C3d were measured in serum samples as described previously.[31] The C3d/C3 ratio was calculated as a measure of complement activation,[32] and is a strong marker for AMD.[29] For the statistical analysis the C3d/C3 ratio underwent natural logarithm transformation.

Statistical analysis

Data were analyzed from September 23, 2014, to November 4, 2015. The odds ratio (OR) of the presence of a rare variant for AMD was calculated by binary logistic regression analysis. Statistical analyses were performed to study differences in age at symptom onset, complement activation levels, family history of AMD, and AMD subtype between carriers and non-carriers of the rare *CFI* p.Gly119Arg, *C9* p.Pro167Ser or *C3* p.Lys155Gln variants. We analyzed the mean values of the continuous traits, complement activation levels, and age at first symptoms using independent sample t-tests and compared the mean value using the Pearson's chi-square test for the other variables. Data were analyzed using SPSS Software version 20.0 (SPSS Inc., Chicago, IL).

RESULTS

The rare variants *CFI* p.Gly119Arg, *C9* p.Pro167Ser and *C3* p.Lys155Gln were observed in 18 individuals in five of the 22 multiplex families with AMD. Although these variants aggregated within these families, they did not segregate completely with the disease (**Figure 1**). The *CFH* p.Arg1210Cys variant was not observed in any of the 22 families.

The *CFI* p.Gly119Arg variant was detected in one family (**Figure 1, family A**). Of the four affected individuals, three were carriers of the *CFI* p.Gly119Arg variant. Affected individual II:4 lacked the *CFI* p.Gly119Arg variant but carried the *CFH* p.Tyr402His risk allele homozygously. The youngest unaffected individual (64 years) carried the risk conferring *CFI* variant. In family B (**Figure 1, family B**), two rare variants, *C9* p.Pro167Ser and *C3* p.Lys155Gln, were identified heterozygously. Although both variants were found only in affected individuals, neither variant segregated fully with the disease phenotype. The *C3* p.Lys155Gln variant was found to cluster in two additional families (**Figure 1, families C and D**). In family C, the *C3* p.Lys155Gln variant was detected in two affected individuals (II:2 and II:4), who also carried the *ARMS2* p.Ala69Ser and *CFH* p.Tyr402His risk alleles homozygously. Individuals II:1 and II:3 were had intermediate AMD without carrying the rare variant in *C3*, and were heterozygous for the common *ARMS2* p.Ala69Ser and *CFH* p.Tyr402His risk alleles. In family D**,** five individuals carried the *C3* p.Lys155Gln variant, of whom four had AMD and one did not (II:8). Individuals II:6 and II:7 carried the *C3* variant and were diagnosed as having intermediate AMD. Their older siblings

II:4 and II:5 who did not carry the rare variant did not develop AMD, although they had a higher genotypic load of the two common variants. In addition to family B, the *C9* p.Pro167Ser variant was also identified in family E (**Figure 1, family E**). Two affected individuals carrying the variant had a more advanced AMD stage than the affected non-carrier family members.

Within the five families, rare variants were detected in 16 affected individuals and 2 unaffected individuals (Table 1). Carrying one of the variants in *CFI, C9* or *C3* resulted in an OR of 7.11 for AMD (95% CI 1.23-40.98; *P* = 0.03).

The age at symptom onset was earlier in affected family members who carried the rare *CFI* p.Gly119Arg, *C9* p.Pro167Ser, or *C3* p.Lys155Gln variants, compared with affected non-carriers (64 versus 69 years; *P* = 0.25)(**Figure 2**). The complement activation level (C3d/C3 ratio) was higher in affected family members who carried a rare variant in a complement gene compared to non-carriers (1.43 and 1.18, respectively; *P* = 0.05)(**Figure 2**). Most patients graded as advanced AMD carried a rare variant. This finding holds true for the single patient with GA and five of the patients with CNV (*P* = 0.17).

Five of 25 affected family members showed an AMD phenotype with reticular pseudodrusen, and all 5 patients carried the rare *CFI* p.Gly119Arg, *C9* p.Pro167Ser, or *C3* p.Lys155Gln variant. Carrying one of these variants was associated with developing reticular pseudodrusen (*P* = 0.02). The p.Pro167Ser variant in the *C9* gene appeared to segregate with the reticular pseudodrusen phenotype in the family E, because individuals II:2 and II:3, who carried the rare variant, showed reticular pseudodrusen, whereas II:1 and II:4 did not. However, the rare variants in the *CFI* and *C3* genes did not segregate with the reticular drusen phenotype. Individual II:1 of family A, and individuals II:2 and II:6 of family D, showed the reticular pseudodrusen phenotype, but this phenotype was not observed in their siblings who carried the same rare variant.

Next, the analyses were replicated in a large case-control EUGENDA cohort, which was genotyped for the rare variants *CFH* p.Arg1210Cys, *CFI* p.Gly119Arg, *C9* p.Pro167Ser, and *C3* p.Lys155Gln. Of the 1589 patients and 1386 controls in the case-control cohort, we identified 91 carriers (5.7%) in the AMD cohort and 43 carriers (3.1%) in the control cohort of the (**Table 1**). The *CFH* p.Arg1210Cys variant was not present in our case-control cohort.

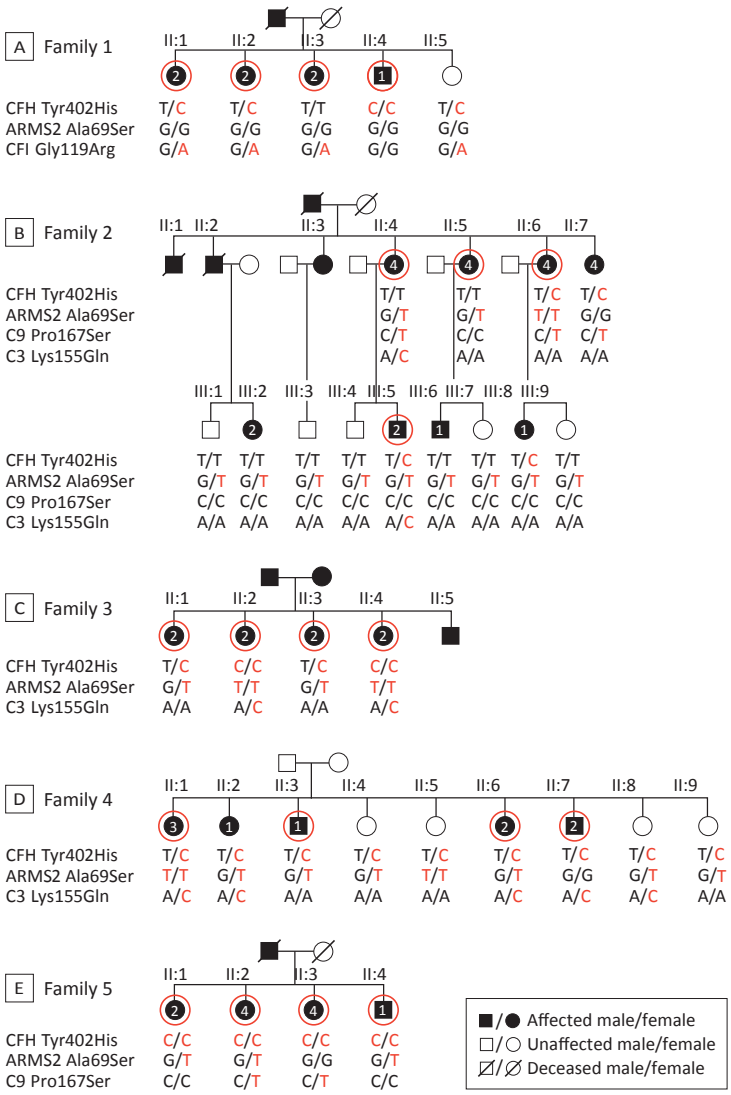

Figure 1: Pedigrees of 5 families with age-related macular degeneration. Pedigrees depict results of segregation analysis of rare and common *CFH* p.Tyr402His and *ARMS2* p.Ala69Ser variants in patients with the rare *CFI* p.Gly119Arg, *C9* p.Pro167Ser, or *C3* p.Lys155Gln variants of complement factor genes. Risk alleles are indicated in red. Affected individuals are assigned grades representing AMD stage (1 indicates early; 2, intermediate; 3, advanced with subfoveal geographic atrophy; and 4, advanced with choroidal neovascularization). Outer circles mark the individuals who underwent whole-exome sequencing.

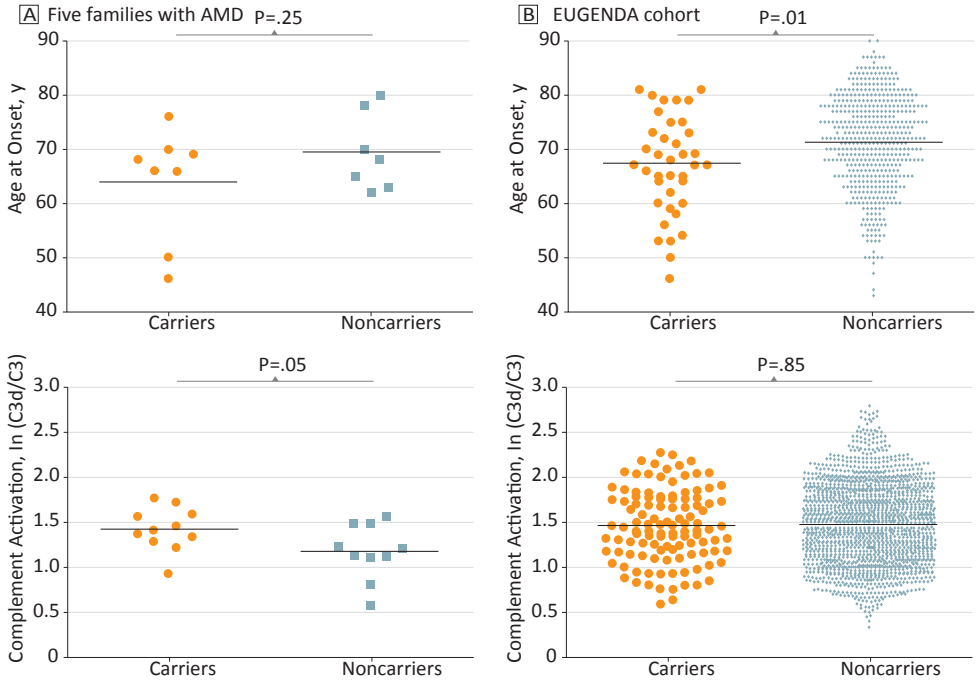

Figure 2: Age at onset and complement activation in carriers versus noncarriers of rare genetic variants. Analysis was performed in the members of 5 families with age-related macular degeneration (AMD) and in AMD cases and controls from the European Genetic Database (EUGENDA) cohort. The difference in age at onset between carriers and noncarriers was not significant in the families (mean [SD] age, 63.9 [10.3] versus 69.4 [7.1] years) but was significant in the EUGENDA cohort (67.4 [8.5] vs 71.3 [8.9] years). The complement activation ratio between carriers and noncarriers in the families (1.43 versus 1.18) and EUGENDA cohort (1.463 vs 1.455) was not significant. Lines indicate mean values. Ln = natural logarithm.

The presence of a rare genetic variant was associated with AMD and conferred an OR of 1.90 (95% CI 1.31-2.75; *P* = 0.001). This association was comparable with the OR for advanced AMD (OR 1.90; 95% CI 1.27-2.85; *P* = 0.002). Separate analyses for each rare variant showed large effect sizes for the *CFI* p.Gly119Arg variant (OR 11.38; 95% CI 1.49-87.06; *P* = 0.003), whereas the effect sizes for the *C9* p.Pro167Ser variant (OR 1.54; 95% CI 0.96-2.45; *P* = 0.07) and the *C3* p.Lys155Gln variant (OR 1.81; 95% CI 0.96-3.44; *P* = 0.06) were smaller (**Table 2**). Patients with AMD who carried the rare *CFI* p.Gly119Arg, *C9* p.Pro167Ser or *C3* p.Lys155Gln variant reported a positive family history for AMD more often than patients with AMD who did not carry these rare variants (35 of 79 [44.9%] versus 367 of 1201 [30.6%], respectively *P* = 0.008). This difference in positive family history was the largest for carriers of the *CFI* p.Gly119Arg variant (58.3% vs 30.6%, *P* = 0.04), followed by variant p.Pro167Ser in *C9* (44.7% vs 30.6%, *P* = 0.04) (**eTable 3**).

Table 1: Clinical characteristics of carriers and noncarriers of the rare variants in the 5 families with AMD and the EUGENDA cohort.

Carrier and disease status	Gender Female No. (%)	Mean (SD) Age, y		Complement activation Mean(SD) C3d/C3 ratio	No. patient graded with GA/CNV	Individuals With Reticular Drusen,No. (%)
		At participation to EUGENDA	At first symptoms			
Families						
Affected carriers (n = 16)	14 (87.5%)	74.9	63.9	1.547 (0.379)	1 and 5	5 (31.3%)
Affected non-carriers (n = 9)	5 (55.6%)	71.4	69.4	1.507 (0.395)	0 and 1	0 (0.0%)
Unaffected carriers (n = 2)	2 (100.0%)	66.0	NA	1.255 (0.371)	NA	0 (0.0%)
Unaffected non-carriers (n = 8)	4 (50.0%)	66.9	NA	1.413 (0.411)	NA	0 (0.0%)
EUGENDA cohort						
Affected carriers (n = 91)	51 (56.0%)	73.7	67.4	1.511 (0.354)	11 and 40	3 (3.3%)
Affected non-carriers (n = 1498)	839 (56.1%)	75.5	71.3	1.457 (0.343)	82 and 795	56 (3.7%)
Unaffected carriers (n = 43)	29 (67.4%)	70.2	NA	1.255 (0.371)	NA	0 (0.0%)
Unaffected non-carriers (n = 1343)	764 (56.9%)	70.4	NA	1.372 (0.362)	NA	0 (0.0%)

Abbreviations: AMD = age-related macular degeneration; CNV = choroidal neovascularization; EUGENDA = European Genetic Database; GA = geographic atrophy; NA = not applicable.

In addition, an earlier age at symptom onset was found in patients with AMD with the rare *CFI* p.Gly119Arg, *C9* p.Pro167Ser, or *C3* p.Lys155Gln variant than in patients who did not carry these rare variants (mean [SD] age, 67.4 [8.5] versus 71.3 [8.9] years, respectively; *P* = .01) (**Table 1 and Figure 2**). In individuals carrying a rare variant, the mean complement activation level (ln C3d/C3) was higher in cases compared with controls (*P* < 0.001). In contrast, the mean C3d/C3 ratio in patients with AMD who carried one of the rare variants was not different from that of non-carriers of these variants (*P* = 0.85)(**Figure 2**). In patients with advanced AMD, the rare *CFI* p.Gly119Arg, *C9* p.Pro167Ser, and *C3* p.Lys155Gln variants were present more often in patients with GA (11 of 93 [11.8%]) than in patients with CNV (40 of 835 [4.8%]; *P* = .04). A reticular pseudodrusen phenotype was present in 59 of 202 AMD patients and none of the 183 controls (**Table 1**). No association was found between the presence of reticular pseudodrusen and the presence of one of these rare variants in the large AMD cohort (*P* = 0.80).

Table 2. Frequencies and effect sizes of the rare variants in the EUGENDA cohort.

Rare variant of Complement Gene	No. (%) of Participants		OR (95% CI)	P-value
	Patients Carriers (n = 1589)	Control Carriers (n = 1386)		
CFI p.Gly119Arg	13 (0.8)	1 (0.1)	11.38 (1.49 - 87.06)	0.003
C9 p.Pro167Ser	49 (3.1)	28 (2.0)	1.353 (0.96 - 2.45)	0.07
C3 p.Lys155Gln	29 (1.8)	14 (1.0)	1.841 (0.96 - 3.44)	0.06
CFH p.Arg1210Cys	0 (0)	0 (0)	-	-

Abbreviations: EUGENDA = European Genetic Database; OR = odds ratio.

DISCUSSION

The development of AMD in densely affected families can be influenced by rare genetic variants, of which four (*CFH* p.Arg1210Cys, *CFI* p.Gly119Arg, *C9* p.Pro167Ser, and *C3* p.Lys155Gln) were previously associated with AMD.[15,18-21] In our EUGENDA case-control cohort, the presence of a variant resulted in an OR of 1.90 for AMD, which is comparable with previously reported effect sizes for the *C9* p.Pro167Ser and *C3* p.Lys155Gln variants.[15,18-21] However, the effect size of the p.Gly119Arg variant in the *CFI* gene was much stronger, with an OR of 11.38, which is in line with those of previous reports (ORs 8.5 and 22.2).[18,33] The *CFH* p.Arg1210Cys variant was previously associated with AMD in North-American cohorts,[15,20] but not in Icelandic[19] and Han Chinese cohorts.[34] The absence of this variant in our AMD case-control cohort may reflect the different distribution of low-frequency alleles among populations.[30]

Almost half of the patients who carried one of the rare *CFI* p.Gly119Arg, *C9* p.Pro167Ser variants or *C3* p.Lys155Gln a positive family history reported for AMD, which has important implications for counseling of these patients and their family members, and underlines the importance of including these rare variants in genetic tests for AMD.

Despite their strong association with AMD in case-control cohorts,[18-21] the *CFI* p.Gly119Arg, *C9* p.Pro167Ser and *C3* p.Lys155Gln variants did not segregate with the disease in the five families in this study. This could point to the contribution of other genetic risk alleles and environmental factors in such multiplex families. Further research is warranted to determine whether additional rare variants aggregate in the remaining 17 families with AMD in this study. Patients who carry a known rare genetic variant differ clinically from patients who do not. We demonstrated that carriers of the rare *CFI* p.Gly119Arg, *C9* p.Pro167Ser or *C3* p.Lys155Gln variants have an onset of symptoms 4-year-earlier, of which the *CFI* p.Gly119Arg variant shows the strongest effect. This finding is comparable to the earlier onset previously described in patients carrying the rare p.Arg1210Cys variant in the *CFH* gene[15] and is in line with the ealier age at symptom onset in patients with familial AMD.[14]

Many of the common genetic variants associated with AMD reside in genes encoding components of the complement cascade,[2,4,35-37] some of which have been associated with increased systemic levels of complement activation and complement components.[25,31,38] Nearly all the recently identified rare variants are located in complement genes,[15,17-21, 39-41] enphasizing the important role of the complement system in the pathophysiology of AMD. Mean complement activation levels were slightly higher in patients with AMD who carry rare variants *CFI*, *C9*, or *C3* gene compared to non-carriers, but this difference in complement activation levels cannot be explained by the presence of the rare variants evaluated in this study. Preventive and therapeutic options inhibiting the complement cascade have been suggested to be effective in treating AMD,[42] but our current results do not support the hypothesis that subjects who carry a rare variant in a complement gene will benefit more from such treatments than non-carriers. However, complement inhibition is a promising target for treatment of GA in AMD,[43] which is more prevalent in affected carriers than non-carriers.

In this study rare variants were more frequently identified in patients with advanced AMD and GA than advanced AMD with CNV. This finding is in line with that of a recent study,[44] which observed higher prevalence of GA among patients carrying the *CFH* p.Arg1210Cys variant. A previously investigation,[14] suggested that additional genetic factors may contribute to the development of GA in familial patients, because a higher prevalence of GA was found in familial than sporadic AMD cases, and siblings are more likely to develop the same advanced AMD subtype as their family proband.[32] The findings of the current study are thus consistent with our suggestion that additional genetic factors play a role in GA development.[14]

From these findings we recommend that patients of European descent, in densely affected families with AMD, undergo screening for rare *CFI* p.Gly119Arg, *C9* p.Pro167Ser and *C3* p.Lys155Gln variants, because these rare variants play a role in more than 20 percent of our screened families. However, these associations may not hold true within populations of other antecedents, because different rare variants in these populations, may play a more important role, such as for example *CFH* p.Arg1210Cys in the North American population.[19]

We observed a higher familial occurrence and an earlier age at onset in the carriers of the rare genetic *CFI* p.Gly119Arg, *C9* p.Pro167Ser and *C3* p.Lys155Gln variants. These findings emphasize the importance of counseling of patients and family members to increase awareness and enable early detection of the disease. Genetic tests for AMD should therefore be designed to detect, in addition to the common variants, the described rare genetic variants, especially in families, because these rare variants contribute to the age at onset and progression of the disease.

Earlier presentation: this study was presented orally during the 'Molecular biology of AMD' session at: the 2015 Annual Meeting of the Association for Research in Vision and Ophthalmology, May 3–7, 2015, Denver, Colorado.

SUPPLEMENTARY INFORMATION

eTable 1. Primer sets used to amplify and Sanger sequence the rare variants in five AMD families

Primer	Primer sequence	Annealing temperature (°C)	Product length (basepairs)
CFI Gly119Arg forward	CGTAAAATGATTGCTTACTATTACTTG	57.1	431
CFI Gly119Arg reverse	TGATGCACATAGTTAATTTTCTTAGG	58.0	
C9 Pro167Ser forward	ACGGTGACATGAACTGAAGC	58.7	388
C9 Pro167Ser reverse	CCAAACTACATCGCCTCTTC	57.4	
*C3*Lys155Gln forward	AGATCCGGAAGCTGGACC	60.2	444
*C3*Lys155Gln reverse	TTGCCTCTCCTAAGCCTGTG	60.5	
CFH Tyr402His forward	TCTTTTTGTGCAAACCTTTGTTAG	59.7	469
CFH Tyr402His reverse	CCATTGGTAAAACAAGGTGACA	59.8	
ARMS2 Ala69Ser forward	ATGCCACCCACAACAACTTT	60.3	403
ARMS2 Ala69Ser reverse	GGTTCTCTCGCTGAGATTCG	60.1	

eTable 2. KASPAR assays used for genotyping the rare variants in EUGENDA case-control cohort

ID	*C9*_rs34882957	*C3*_rs147859257
Primer_AlleleFAM	CATTGTCAAAAGGTGTGCTTAGGGA	GGATCTTCACCGTCAACCACC
Primer_AlleleHEX	GTCAAAAGGTGTGCTTAGGGG	CGGATCTTCACCGTCAACCACA
Primer_Common	TTCTCAGGATCAACATTTTAGGGATGGAT	ACCGTCCGGCCCACGGGTA

eTable 3. Quantitative traits for each of the rare variants in the EUGENDA case-control cohort

Rare variant	Mean age at onset in carriers vs non-carriers (years)	*P*-value	Familial occurance in carriers vs non-carriers (%)	*P*-value	Mean complement ratio in carriers vs non-carriers	*P*-value
CFI p.Gly119Arg	58.7 vs 71.3	0.005	58.3 vs 30.6	0.038	1.670 vs 1.463	0.125
C9 p.Pro167Ser	69.1 vs 71.3	0.225	44.7 vs 30.6	0.040	1.442 vs 1.463	0.325
C3 p.Lys155Gln	66.5 vs 71.3	0.127	36.6 vs 30.6	0.556	1.486 vs 1.463	0.745

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Maartje J. Geerlings
Mariann Kremlitzka
Bjorn Bakker
Sara C. Nilsson
Nicole T. Saksens
Yara T. Lechanteur
Marc Pauper
Jordi Corominas
Sascha Fauser
Carel B. Hoyng
Anna M. Blom
Eiko K. de Jong
Anneke I. den Hollander

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3

THE FUNCTIONAL EFFECT OF RARE VARIANTS IN COMPLEMENT GENES ON C3B DEGRADATION IN PATIENTS WITH AGE-RELATED MACULAR DEGENERATION

ABSTRACT

Importance: In age-related macular degeneration (AMD), rare variants in the complement system have been described, but their functional consequences remain largely unexplored.

Objectives: To identify new rare variants in complement genes and determine the functional effect of identified variants on complement levels and complement regulation in serum samples from carriers and noncarriers.

Design, setting and participants: This study evaluated affected (n = 114) and unaffected (n = 60) members of 22 families with AMD and a case-control cohort consisting of 1831 unrelated patients with AMD and 1367 control individuals from the European Genetic Database from March 29, 2006, to April 26, 2013, in Nijmegen, the Netherlands, and Cologne, Germany. Exome sequencing data of families were filtered for rare variants in the complement factor H (*CFH*), complement factor I (*CFI*), complement C9 (*C9*), and complement C3 (*C3*) genes. The case-control cohort was genotyped with allele-specific assays. Serum samples were obtained from carriers of identified variants (n = 177) and age-matched noncarriers (n = 157). Serum concentrations of factor H (FH), factor I (FI), C9, and C3 were measured, and C3b degradation ability was determined.

Main Outcome Measures: Association of rare variants in the CFH, CFI, C9, and C3 genes with AMD, serum levels of corresponding proteins, and C3b degradation ability of CFH and CFI variant carriers.

Results: The 1831 unrelated patients with AMD had a mean (SD) age of 75.0 (9.4) years, and 60.5% were female. The 1367 unrelated control participants had a mean (SD) age of 70.4 (7.0), and 58.7% were female. All individuals were of European descent. Rare variants in *CFH*, *CFI*, *C9*, and *C3* contributed to an increased risk of developing AMD (odds ratio, 2.04; 95% CI, 1.47-2.82; P < .001). *CFI* carriers had decreased median FI serum levels (18.2 µg/mL in p.Gly119Arg carriers and 16.2 µg/mL in p.Leu131Arg carriers versus 27.2 and 30.4 µg/mL in noncarrier cases and controls, respectively; both P < .001). Elevated C9 levels were observed in p.Pro167Ser carriers (10.7 µg/mL versus 6.6 and 6.1 µg/mL in noncarrier cases and controls, respectively; P < .001). The median FH serum levels were 299.4 µg/mL for *CFH* p.Arg175Gln and 266.3 µg/mL for *CFH* p.Ser193Leu carriers versus 302.4 and 283.0 µg/mL for noncarrier cases and controls, respectively. The median C3 serum levels were 943.2 µg/mL for *C3* p.Arg161Trp and 946.7 µg/mL for *C3* p.Lys155Gln carriers versus 874.0 and 946.7 µg/mL for noncarrier cases and controls, respectively. The FH and FI levels correlated with C3b degradation in noncarriers (R^2 = 0.35 and R^2 = 0.31, respectively; both P < .001).

Conclusions and relevance: Reduced serum levels were associated with C3b degradation in carriers of *CFI* but not *CFH* variants, suggesting that *CFH* variants affect functional activity of FH rather than serum levels. Carriers of *CFH* (p.Arg175Gln and p.Ser193Leu) and *CFI* (p.Gly119Arg and p.Leu131Arg) variants have an impaired ability to regulate complement activation and may benefit more from complement-inhibiting therapy than patients with AMD in general.

INTRODUCTION

Age-related macular degeneration (AMD) is caused by a combination of environmental and genetic factors. Although aging and smoking confer the strongest non-genetic risk, genetic alterations account for 45% to 70% of the variability in disease risk.[1] Genetically, AMD is heterogeneous, with 34 genomic loci implicated in disease pathogenesis. Susceptibility genes that reside in these loci are grouped into 4 main pathways: (1) complement system, (2) high-density lipoprotein metabolism, (3) angiogenesis, and (4) extracellular matrix remodeling.[2,3]
The complement system is part of the innate immune system, and tight regulation of this system is needed to protect the body's own cells from tissue damage. The central component of the system is C3, which is cleaved into C3b and C3a. C3b is a crucial component of C3 and C5 convertases that catalyze further steps in the cascade. The final step is the formation of the membrane attack complex, which includes several copies of C9. Factor H (FH) is one of the main inhibitors of complement through binding of C3b and aiding its degradation by serine protease factor I (FI).[4,5]

In AMD, the complement system is highly burdened by genetic variations.[6] Most of these genetic variants are relatively common in the population and have a modest to low effect on AMD development.[3] Recently, rare genetic variants (defined by a minor allele frequency <1%) in the complement system were also described to play an important role in AMD. Such rare variants were described in the complement factor H (*CFH*) (NM_000186),[7-10] complement factor I (*CFI*) (NM_000204),[11,12] complement factor 9 (*C9*) (NM_001737),[12] and complement factor 3 (*C3*) (NM_000064) genes.[12-15] Carriers of these rare genetic variants presented with a younger age at disease onset and more often progressed to end-stage AMD compared with noncarriers.[10,16-19]

Only a limited number of studies[10-12,18-21] have investigated functional effects of rare variants on activity of the affected protein and the complement system overall. The reported effects of rare CFH variants on FH levels are inconsistent. Although one study[19] reported reduced serum FH levels in rare variants carriers, others[10,20] did not observe this effect. Lower FI serum and plasma levels were found in carriers of *CFI* variants compared with controls.[11,18] In addition, the *CFI* variant p.Gly119Arg resulted in a lower ability to degrade C3b.[11] Similarly, carriers of the rare variant p.Lys155Gln in *C3* had reduced C3b cleavage.[12] The *C3* variant p.Arg161Trp was reported to affect the ability of FH to inhibit C3 convertase.[21] *C9* variants were previously associated with AMD[12,22]; however, the functional effect of these variants has not been studied.

In this study, we aimed to identify novel rare genetic variants in complement genes previously associated with AMD. We intended to determine the effect of rare genetic variants on levels of complement components in serum and analyze the ability to degrade C3b in serum samples from rare variant carriers compared with noncarriers.

METHODS

Participants

We evaluated 22 severely affected AMD families with at least 4 affected siblings, resulting in 114 affected and 60 unaffected family members, from March 29, 2006, to April 26, 2013, in Nijmegen, the Netherlands, and Cologne, Germany. In addition, 1831 unrelated patients with AMD and 1367 unrelated control individuals from the European Genetic Database (EUGENDA) were studied. Control individuals were 60 years or older. All patients underwent clinical evaluation and were graded for AMD according to the Cologne Image Reading Center protocol.[17,23] Serum samples were obtained by a standard coagulation and centrifugation protocol, after which they were stored at –80°C within 1 hour after collection. Genomic DNA was isolated from peripheral blood samples according to standard procedures. This study was approved by local ethics committees on research involving human subjects, namely, the Commissie Mensgebonden Onderzoek Regio Arnhem- Nijmegen and the local committee of University Hospital Cologne, and met the criteria of the Declaration of Helsinki.[24] Before enrollment in EUGENDA, all participants provided written informed consent and were assigned a database identifier code for anonymization.

Genetic Analysis

Whole exome sequencing analysis was implemented to uncover the coding regions (the Supplementary information) of selected complement genes previously reported to harbor rare variants associated with AMD, namely, *CFH, CFI, C9*, and *C3*.[7-15] From the candidate genes, we selected variants that would induce an amino acid change. Frequency filters from the public databases 1000 Genomes Project and Exome Variant Server database ensured selection of rare variants only. Variants with a minor allele frequency less than 1% were considered rare. Variants found in multiple individuals were selected for Sanger sequencing as confirmation and segregation. Primer sets used for Sanger sequencing were designed manually using Primer3Plus.[25] Predicted effect of each variation was examined using PolyPhen2 and SIFT (Sorting Intolerant From Tolerant).[26,27]

Genotyping

Genotyping of rare genetic variants *CFH* p.Ser193Leu, *CFH* p.Arg175Gln, *CFI* p.Pro553Ser, *CFI* p.Leu131Arg, *C9* p.Arg118Trp, and *C3* p.Arg161Trp was performed for participants of the EUGENDA case-control cohort by custom-made competitive allele-specific polymerase chain reaction assays (Kompetitive Allele Specific Single-Nucleotide Polymorphism Genotyping System; LGC Ltd) according to the manufacturer's recommendations.

Selection of Serum Samples

We collected serum samples of rare variant carriers (n = 157) and available family members (n = 93). Two comparison groups with similar mean age had available serum samples: (1) 77 patients with AMD who did not carry any of the selected variants and (2) 80 control individuals who did not have any of the selected variants. The total number of serum samples encompassed 407, of whom 201 individuals carry a rare variant.

Functional Analysis

The concentrations of FH, FI, C9, and C3 in serum samples were measured by enzyme-linked immunosorbent assay in triplicate.[28] In addition, the degradation of C3b in fluid phase was analyzed to assess how *CFH* and *CFI* variants affect the proteins' ability to degrade C3b in the fluid phase. Details are in **the supplementary information**.

Statistical Analysis

Because of the low frequency of rare variants, asymptotic statistics can be inaccurate. Therefore, we used exact statistics to test association for individual variants. This analysis in- cluded an aggregate meta-analysis, which included close family members. To include the first-degree siblings (second- degree siblings for families A and E), we calculated statistical significance using a binominal distribution. When family members were included, the chance for siblings to inherit similar rare genetic variation was 50%. The calculation of this statistical aggregation score has been replicated as described in detail by Raychaudhuri et al[7] and a previously rare variant analysis.[12]

Serum levels and carrier status were analyzed using Kruskal-Wallis with the Dunn post hoc comparison adjustment. Within figures, median values with interquartile ranges are depicted, and differences at $P < .05$ were considered statistically significant. For correlations, the covariates AMD status and rare variant status were included. Spearman ρ correlation coefficient was used for nonparametric correlations, and P values were evaluated using Bonferroni correction ($P < .007$ was considered significant).

RESULTS

The 1831 unrelated patients with AMD had a mean (SD) age of 75.0 (9.4) years, and 60.5% were female. The 1367 unrelated controls had a mean (SD) age of 70.4 (7.0), and 58.7% were female. All individuals were from European descent. We found *CFI* p.Gly119Arg, *C9* p.Pro167Ser, and *C3* p.Lys155Gln, which have been previously associated with AMD,[7,11-14] and we described their familial segregation in detail previously.[17] We identified 6 additional rare genetic variants, namely, *CFH* p.Ser193Leu, *CFH* p.Arg175Gln, *CFI* p.Pro553Ser, *CFI* p.Leu131Arg,

C9 p.Arg118Trp, and *C3* p.Arg161Trp (**Table 1**). Although the identified variants were highly prevalent within these families, a perfect segregation with disease phenotype was not observed (**eFigure in the Supplement**).

The 6 new variants clustered in 5 AMD families (families A, B, D, E, and F). Furthermore, we identified 2 smaller families, both consisting of 3 siblings, carrying the same variants (families C [*CFH* p.Ser193Leu] and G [*C3* p.Arg161Trp]). Newly identified rare variants were present more frequently in affected members compared with unaffected individuals (28 of 37 [75.7%] and 4 of 10 [40.0%], respectively; P = .07).

Next, we investigated whether the rare variants identified in our families were associated with AMD in a case-control cohort of 1831 patients with AMD and 1367 control individuals. We identified 194 carriers of novel and previously identified variants in complement genes *CFH*, *CFI*, *C9*, or *C3*. Carrying one of these rare variants was significantly associated with AMD status because 139 carriers (72.4%) were AMD case patients and 53 (27.6%) were control individuals (odds ratio, 2.04; 95% CI, 1.47-2.82; P < .001). In the meta-analysis, which combined results of the family and case-control cohorts, all variants had a nominal association with AMD (**Table 2 and eTable 1 in the Supplement**).

To determine the effect of newly identified rare variants (*CFH* p.Ser193Leu, *CFH* p.Arg175Gln, *CFI* p.Pro553Ser, *CFI* p.Leu131Arg, *C9* p.Arg118Trp, and *C3* p.Arg161Trp) and of previously identified variants (*CFI* p.Gly119Arg, *C9* p.Pro167Ser, and *C3* p.Lys155Gln)[17] on protein expression, we performed serum measurements. Levels of FH, FI, C3, and C9 were determined by enzyme-linked immunosorbent assay in serum samples of 314 individuals, of which 157 carried a rare variant. Carriers of different variants were grouped per gene and included both patients with AMD and controls. To assess differences between cases and con- trols, noncarriers were split based on AMD status (**Figure 1**). Significant differences in serum levels of FI and C9 were observed (**eTable 2 in the Supplement**).

Table 1: Rare variants in complement genes identified in families with age-related macular degeneration

Gene	Complementary DNA Change	Protein change	rs-number	EVS,%	1000, %	phyloP Score*	Grantham Score*	SIFT**	PP2**	Family	Source
CFH	578C>T	Ser193Leu	-	-	-	2.433	145	T	D	B and C	Duvvari et al., [8] 2015
CFH	524G>A	Arg175Gln	rs139360826	-	-	-0.395	43	T	B	E	Duvvari et al., [8] 2015
CFI	1657C>T	Pro553Ser	rs113460688	0.14	0.05	0.443	74	T	B	F	Bienaime et al,[29] 2010; Kavanagh et al,[30] 2012; Roumenina et al,[31] 2012
CFI	392T>G	Leu131Arg	-	-	-	2.116	102	D	D	A	Novel
C9	352 C>T	Arg118Trp	rs147701327	0.05	-	0.788	101	D	P	D	Novel
C3	481 C>T	Arg161Trp	rs776423109	-	-	0.151	101	D	D	E and G	Duvvari et al [15] 2014

Abbreviations: B, benign; C3, complement factor 3 gene; C9, complement factor 9 gene; CFH, complement factor H gene; CFI, complement factor I gene; D, damaging; EVS, Exome Variant Server; NA, not applicable; P, pathogenic; PP2, PolyPhen2; rs, reference single-nucleotide polymorphism identification; SIFT, Sorting Intolerant From Tolerant. [a] Higher PhyloP (range, –14 to 6.4) and Grantham (range, 0-215) scores correlate with a higher conservation.

Table 2: Carriers and noncarriers of rare variants in families with AMD and in the case-control cohort

Gene	Proband and first degree relatives*				Case-Control Cohort				Allelic OR[b]	P value[c]
	Heterozygous carriers		Non-carriers		Heterozygous carriers		Noncarriers			
	Control	AMD	Control	AMD	Control	AMD	Control	AMD		
CFH p.Ser193Leu	0	5	1	2	0	5	1367	1823	NA	0.0057
CFH p.Arg175Gln	0	8	1	3	1	2	1366	1826	1.496	0.0443
CFI p.Pro553Ser	0	3	0	3	2	10	1362	1816	3.742	0.0378
CFI p.Leu131Arg	1	4	5	4	0	4	1366	1823	NA	0.0227
C9 p.Arg118Trp	0	3	1	0	2	3	1364	1824	1.122	0.0377
C3 p.Arg161Trp	1	9	1	4	0	4	1367	1828	NA	0.0104

Abbreviations: AMD, age-related macular degeneration; C3, complement factor 3 gene; C9, complement factor 9 gene; CFH, complement factor H gene; CFI, complement factor I gene; NA, not applicable; OR, odds ratio. [a] Families A and E are extended pedigrees that also include second-degree family members. [b] The allelic OR could not be calculated for variants that were found only in cases (not applicable). [c] Calculated with 1-tailed t test.

Carriers of *CFI* p.Gly119Arg had a significantly decreased median serum FI level (18.2 µg/mL) compared with noncarrier cases and controls (27.2 and 30.4 µg/mL, both P < .001), as did carriers of *CFI* p.Leu131Arg (16.2 µg/mL) versus noncarrier cases (27.2 µg/mL, P = .005) and controls (30.4 µg/mL, P = .001) (**Figure 1B**). Carriers of *C9* p.Pro167Ser had an elevated C9 serum level (10.7 µg/mL) compared with noncarriers (6.6 µg/mL in cases and 6.1 µg/mL in controls, P < .001) (**Figure 1C**). Median FI serum levels for non- carriers were 27.2 and 30.4 µg/mL (cases and controls, respectively). Median C9 serum levels for noncarriers were 6.6 and 6.1 µg/mL (cases and controls, respectively). The median FH serum levels were 299.4 µg/mL for *CFH* p.Arg175Gln and 266.3 µg/mL for *CFH* p.Ser193Leu carriers versus 302.4 and 283.0 µg/mL for noncarrier cases and controls, respectively. The median C3 serum levels were 943.2 µg/mL for *C3* p.Arg161Trp and 946.7 µg/mL for *C3* p.Lys155Gln carriers versus 874.0 and 946.7 µg/mL for noncarrier cases and controls, respectively (**Figure 1A and D and eTable 2 in the Supplement**).

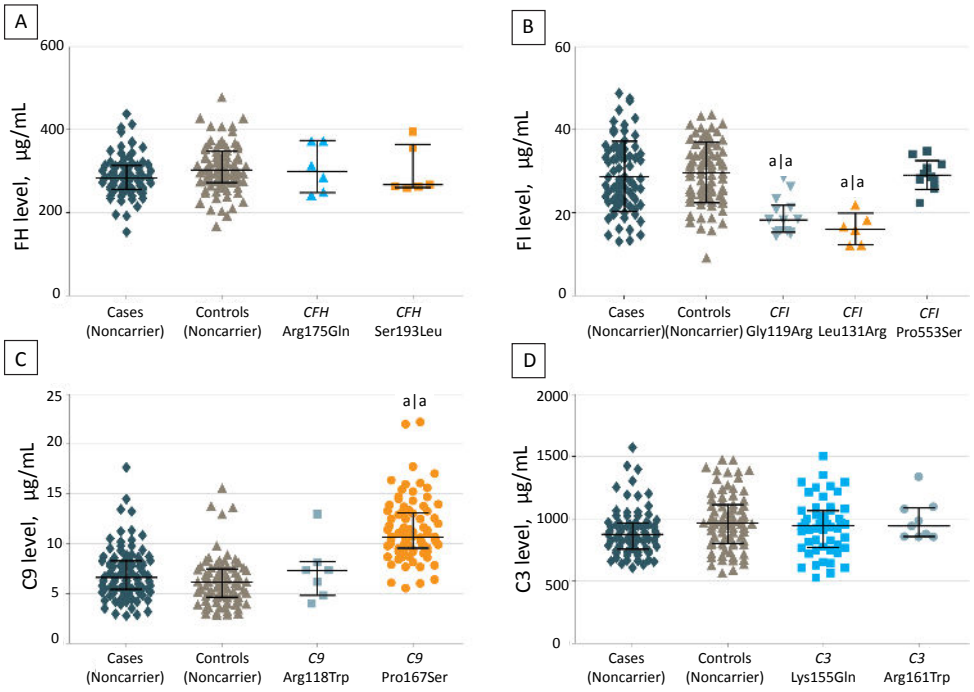


Figure 1: Serum levels of factor H (FH), factor I (FI), C9, and C3 in carriers and noncarriers. Serum levels of FH (A), FI (B), C9 (C), and C3 (D) measured in carriers and noncarriers in corresponding genes. Significance values are on the left (versus noncarrier cases) and right (versus noncarrier controls). Lines indicate median; error bars, interquartile range. *C3* indicates complement 3 gene; *C9*, complement 9 gene; *CFH*, complement factor H gene; and *CFI*, complement factor I gene. [a] P < .001.

Next, we assessed C3b degradation in serum samples from individuals carrying *CFH* or *CFI* mutations and compared them with noncarriers. As illustrated in Figure 2, carriers of rare variants in *CFH* and *CFI* had a lower capacity to degrade C3b when compared with noncarriers, except for *CFI* p.Pro553Ser carriers (eTable 3 in the Supplement).

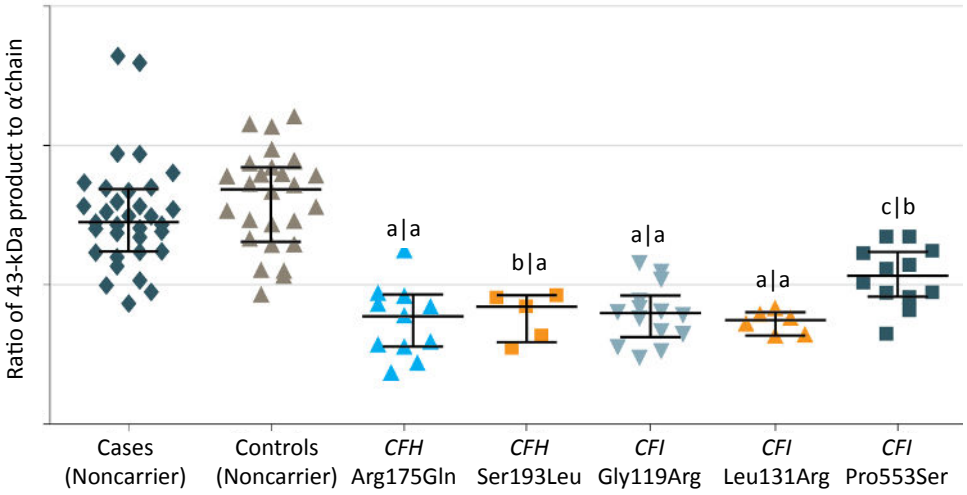


Figure 2: C3b Degradation Assay in Serum Samples of Complement Factor H (CFH) and Complement Factor I (CFI) Gene Variant Carriers. C3b degradation depicted as ratio of 43-kDa degradation product over the α′ chain. Significance values are on the left (versus noncarrier cases) and right (versus noncarrier controls). Lines indicate median; error bars, interquartile range. [a] P < .001 [b] P < .01. [c] P = .39.

Finally, we determined whether FH and FI levels affect an individual's ability to degrade C3b irrespective of rare variant status (**Figure 3**). Linear regression revealed a positive correlation between both FH and FI serum levels with C3b degradation ratios in noncarrier cases and controls (r^2 = 0.34 for FH serum levels and r^2 = 0.31 for F1 serum levels; P < .001). Carriers of *CFH* and *CFI* variants were plotted in these graphs, revealing lower serum concentrations and/or a reduced ability to degrade C3b.

DISCUSSION

We identified 6 new rare variants in complement genes in 5 of 22 densely affected families with AMD. Serum measurements revealed altered serum levels for individuals carrying some of these variants compared with controls. In addition, serum samples from carriers of rare variants in *CFH* and *CFI* revealed a diminished ability to degrade C3b, suggesting that the variants result in impaired complement regulation.

To our knowledge, this is the first description of variants *CFI* p.Leu131Arg and *C9* p.Arg118Trp in the literature. Variants p.Ser193Leu and p.Arg175Gln in *CFH* were both previously identified by our group.[8] *CFI* p.Pro553Ser has been described earlier in atypical hemolytic uremic syndrome (aHUS), a severe rare renal disease, but was also noted as a possible risk variant for AMD.[12,18,29,30] The p.Arg161Trp variant in *C3*, another aHUS variant,[31] was previously reported.[15]

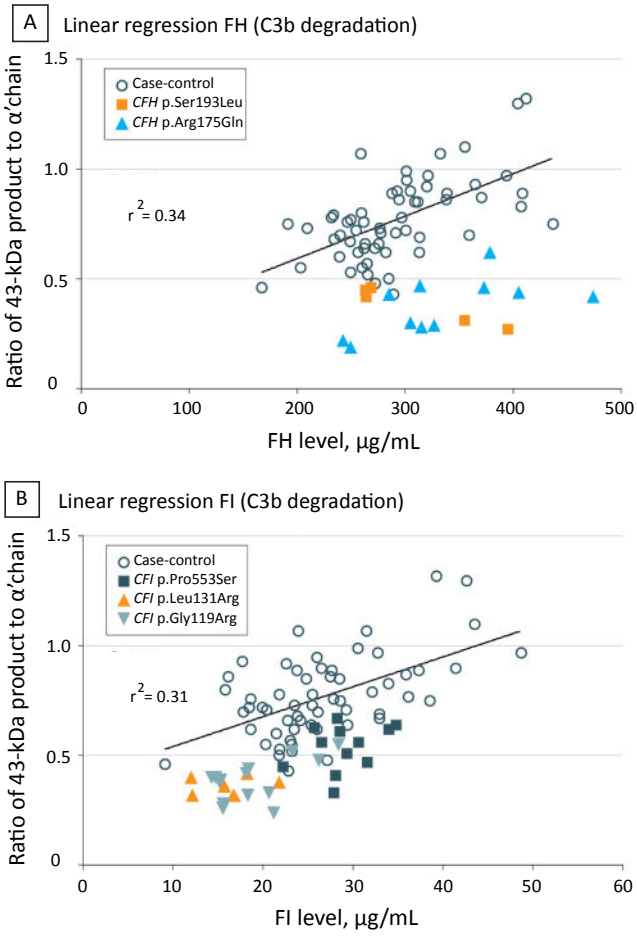


Figure 3. Correlation Between Factor H (FH) and Factor I (FI) Serum Levels With the Ability to Degrade C3b. Correlation between the FH (A) and FI (B) serum levels and the ability to degrade C3b. Complement factor H (*CFH*) gene carriers had normal serum levels but functional defect. Complement factor I (*CFI*) gene variants had reduced serum levels.

In our case-control cohort, the newly and previously described rare variants were predominantly found in cases and contributed to an increased risk of developing AMD. Similarly, the carrier status within the families was higher in cases compared with unaffected family members. Even though the variants did not perfectly segregate with the AMD phenotype, the combined P value of the case-control and family cohorts supports an association of these rare variants with AMD. Our findings further strengthen the notion that rare variants in complement genes play an important role in AMD development and that family studies are a useful approach to identify rare variants.[3,10,32]

By analyzing the effect of rare variants on protein expression, we found that carriers of *CFH* mutations have normal serum FH concentration. Although no functional analysis has been described for either of these variants, a previous study[19] measured FH levels in five patients with AMD carrying other rare variants in *CFH* (p.Cys192Phe, p.Tyr277*, p.Cys431Ser, two splice site variants). Lower median FH concentrations were observed in carriers compared with noncarriers. In another study, ten carriers of *CFH* variants p.Arg53Cys and p.Asp90Gly were found to have normal FH concentrations. Similarly, measurement of serum levels of *CFH* p.Gln950His carriers revealed FH levels within the reference range.[20] These results indicate that not all rare variants in *CFH* lead to lower FH levels. Variants group into two major mutation types. Type 1 mutations cause low protein levels as a result of misfolding or degradation, whereas type 2 mutations result in reduced functionality with normal protein levels. The *CFH* variants identified in our study (*CFH* p.Ser193Leu and p.Arg175Gln) are most likely type 2.

The FI levels were measured in the serum of carriers of *CFI* p.Gly119Arg, p.Leu131Arg, and p.Pro553Ser. It was previously reported that individuals with advanced AMD carrying *CFI* variants have reduced FI concentration.[18] In particular, p.Gly119Arg had high odds ratios for AMD and significantly reduced FI levels in plasma.[11] We confirm this finding and report that novel variant p.Leu131Arg in *CFI* also leads to a strong reduction of FI serum levels. The third variant, *CFI* p.Pro553Ser (ten cases and two controls), did not alter FI levels compared with noncarriers, which is consistent with previous studies.[12,18]

C3 levels of carriers of *C3* p.Lys155Gln or p.Arg161Trp variants were normal compared with noncarriers, in line with a previous report.[33] Another study[12] found that carriers of rare variant p.Lys155Gln failed to degrade C3b properly and hypothesized that this was caused by reduced binding to FH. Although *C3* p.Lys155Glnis was highly associated with AMD, median serum level of *C3* Lys155Gln did not differ from that of noncarriers, supporting that the variant influences protein functionality rather than level. In aHUS, p.Arg161Trp has been described to be pathogenic because of a hyperactive C3 convertase formation attributable to increased binding to factor B, accompanied by increased C3a, C5a, and membrane attack complex. The p.Arg161Trp variant leads to reduced binding to FI cofactors, such as FH.[21,31,34,35] Although *C3*

variants result in a lower C3 level in most aHUS and AMD patients (70%-80%), very low C3 levels are observed only in patients with homozygous *CFH* or gain-of-function mutations in *CFB* or *C3*.[21,33,36] One individual in family E carrying the p.Arg161Trp (indicated by an asterisk) reported reduced renal function attributable to hypertension and urolithiasis.

To our knowledge, we are the first to report significantly elevated C9 serum levels in carriers of *C9* p.Pro167Ser in AMD. We hypothesize this increased C9 level results from elevated complement activation in patients with AMD, which, through lysis of the target cells, may directly contribute to retinal destruction observed in the disease pathogenesis. For *C9* only two variants have been described in AMD: *C9* p.Pro167Ser[12] and p.Arg95*,[22] the latter being inherent to Asian populations. Our study indicates that other rare variants in the *C9* gene, such as p.Arg118Trp, are also associated with AMD, although this variant did not affect C9 serum concentration.

In this study, all carriers of rare variants in *CFH* and *CFI* had reduced ability to degrade C3b compared with noncarriers. Furthermore, carriers of *CFI* variants (p.Gly119Arg and p.Leu131Arg) had decreased FI serum level. A previous study,[11] using recombinant FI (p.Gly119Arg), reported that both expression and secretion of mutant protein were reduced compared with wild-type protein. Consequently, impaired levels led to reduced C3b degradation. The FH levels remained stable, suggesting that *CFH* variants affect complement activation independent of FH serum levels by its inability to properly serve as a cofactor in the cleavage of C3b to inactive C3b. This finding might be explained by the variants' location at the N-terminus, where a C3b-binding site is located.[37]

We detected a natural correlation of FH and FI levels with the ability to degrade C3b in noncarrier individuals. Carriers of rare variants in *CFH* and *CFI* group outside the linear curve of noncarriers. This finding suggests that carriers of rare variants in the *CFH* and *CFI* genes have a decreased ability to degrade C3b and thus higher levels of complement activation compared with noncarriers and may benefit more from complement-inhibiting therapy than patients with AMD in general.

Several clinical trials are currently evaluating complement- inhibiting treatments in AMD,[38] and two clinical trials have been completed. The Complement Inhibition with Eculizumab for the Treatment of Nonexudative Age-Related Macular Degeneration (COMPLETE) study involved eculizumab, an anti- body that binds to C5 and inhibits cleavage of C5 to C5a.[39] The trial results suggested eculizumab was not effective in treating AMD because the growth of geographic atrophy (advanced AMD) did not decrease after 6 months of treatment. The Safety, Tolerability, and Evidence of Activity of FCFD4514S Administered Monthly or Every Other Month to Patients With Geographic Atrophy (MAHALO) study with lampalizumab, an antibody directed

against complement factor D, which is a rate- limiting enzyme involved in the activation of the alternative pathway,[40] had promising results because progression of the geographic atrophy lesion had a 20% reduction after 18 months of treatment. Lampalizumab has been suggested to be most effective in a subpopulation of patients because an even higher reduction rate was seen in patients with a specific *CFI* genotype.[40] Selection of such patients would lead to more effective clinical trials, requiring smaller patient groups to re- veal an effect of the drug being tested. Screening of individuals for genetic (eg, rare variants in *CFH* and *CFI*) or serum (eg, reduced FI levels) biomarkers will enable treatment in an early phase of the disease, before substantial tissue damage has occurred. Personalized treatment could be provided for patients with rare genetic variants in the *CFH* and *CFI* genes or reduced FI levels, linked to the functional inability to degrade C3b efficiently, and act on this effect by using complement inhibitors.

Limitations

A limitation of the study is that functional effects of variants were only assessed for those variants that were significantly associated with AMD. Private variants were not included in this study. Enlarging the study cohort would improve the power to detect rare variants.

CONCLUSIONS

We identified multiple rare variants in complement genes encoding FH, FI, C9, or C3. Carriers of *CFI* (p.Gly119Arg; p.Leu131Arg) had decreased FI levels, whereas individuals with the *C9* p.Pro167Ser variant had elevated serum concentrations compared with noncarriers. Carrying a *CFH* or *C3* variant did not change FH or C3 levels. Carriers of rare variants in *CFH* and *CFI* had a reduced ability to degrade C3b compared with noncarriers. For *CFI*, this effect was linked to reduced serum FI levels, but *CFH* affects C3b degradation independent of FH serum levels. Carriers of rare variants in *CFI* and *CFH* are less able to inhibit complement activation and may benefit more from complement-inhibiting therapy than patients with AMD in general. Our results suggest that patients with AMD should be screened using a functional complement assay and should be tested for rare genetic variants and corresponding serum levels to apply the most proper therapeutic regimen for disease treatment.

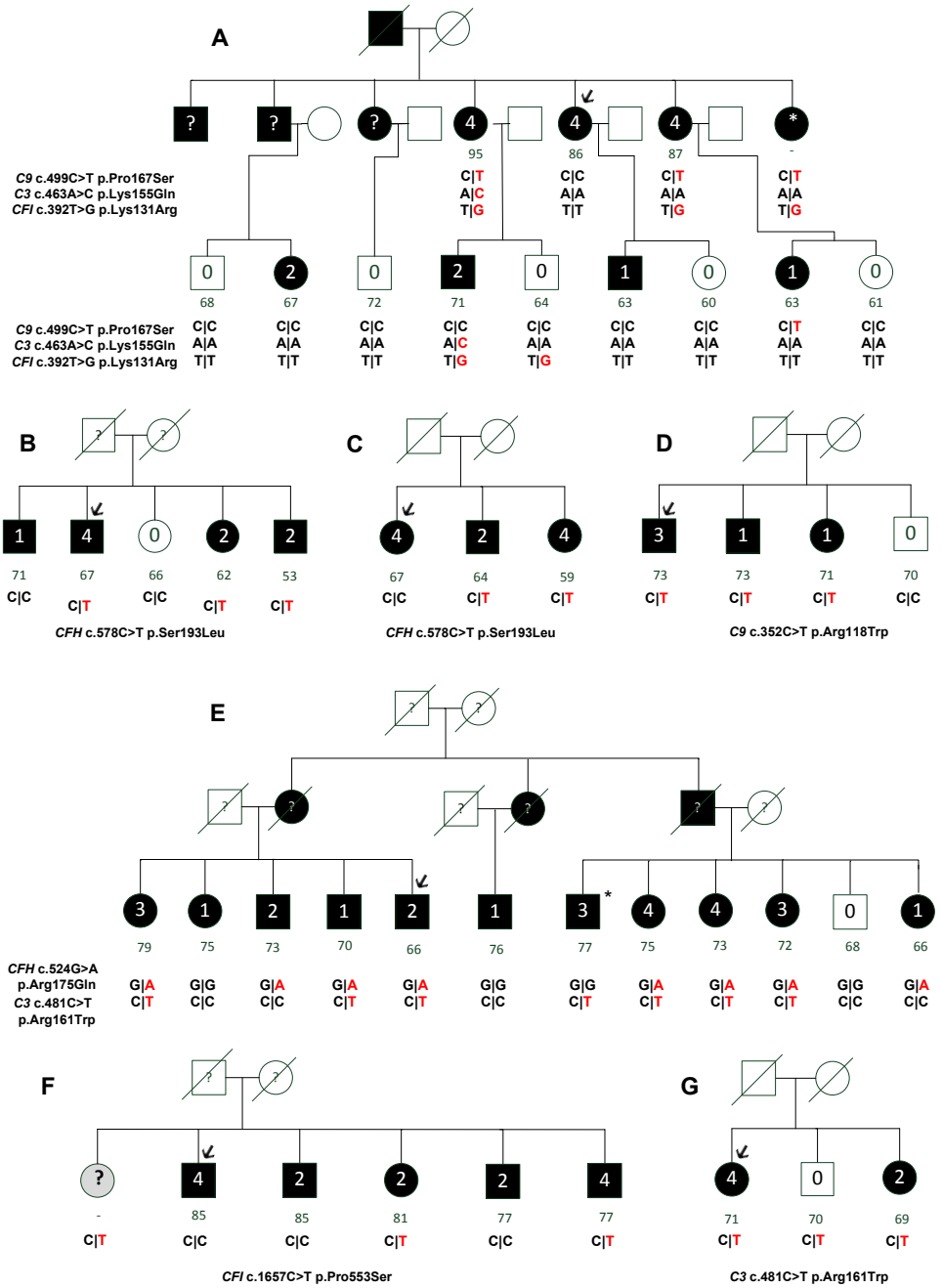
Earlier presentation: this study was presented as a poster during the 'Complex traits and Polygenic Disorders' session at: the 2016 American Society of Human Genetics annual meeting, October 18–22, 2016, Vancouver, Canada.

SUPPLEMENTARY INFORMATION

Genetic analysis and genotyping: Exome capture was achieved using the Nimblegen SeqCap EZ V2 kit (Roche Nimblegen, Inc., Madison, WI), on Illumina HiSeq2000 sequencer using Illumina TruSeq V3 chemistry (Illumina, Inc., San Diego, CA). Downstream analyses included demultiplexing (CASAVA software, Illumina) and alignment to the hg19 reference genome (Genome Reference Consortium Human Reference 37) by Burrows-Wheeler alignment tool. Alignments were sorted by Picard (http://broadinstitute.github.io/picard) and subsequently processed by GATK (Indel Realignment and Base-Quality Score Recalibration). PCR duplicates were marked by Picard, Mean Depth of Coverage was determined using GATK, and Freemix values were estimated through verifyBAMid. Samples that passed technical QC metrics were genotyped to gVCF level through GATKs HaplotypeCaller. Insertions, deletions, and single nucleotide variants were filtered separately using GATKs Variant-Quality Score Recalibration, and annotated using ANNOVAR. Quality filters established high quality annotation with sufficient reads (number or reads ≥10, and percentage of variation ≥20%).

ELISA: Maxisorp Nunc Immunoplates (Nunc) were coated overnight either with 5 µg/mL anti-human FH (Abcam), or 10 µg/mL anti-human FI (generated in house), or anti-human C3 (1:1000 dilution, Dako) or 5 µg/mL anti-human C9 (Hycult Biotech) in 50 mM sodium carbonate (pH 9.6) at 4 °C. After blocking with Quench solution (50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 2 mM $CaCl_2$, 0.1% Tween 20 and 3% fish gelatin), plates were incubated with serum samples for 1h at 37^{0}C in the following dilutions: FH (1:3000), FI (1:1000), C3 (1:30.000) and C9 (1:100). As standards, recombinant wild-type proteins were used. After incubation, bound proteins were detected using goat anti-human FH (Abcam, 1 µg/mL), or anti-human FI (Quidel, 1:2000), or anti-human C3 (Quidel, 1:1000), or anti-human C9 (Complement Technologies, 1:4000) followed by rabbit antibody to goat immunoglobulin conjugated with horseradish peroxidase (Dako; 1:2000 dilution for FI, C3 and C9 or 1:5000 dilution for FH). As substrate, 1,2-phenylenediamine dihydrochloride (Dako) was used.

Degradation of C3b in fluid phase: serum samples were diluted 50X in 50 mM Tris-HCl and 150 mM NaCl, pH 8.0, and mixed with trace amounts of AlexaFluor 647-labeled C3b (C3b was purchased from Complement Technologies and labeled according to the manufacturer's instructions; Pierce). A mixture of 20 µg/ml plasma-purified FI, 20 µg/ml plasma-purified FH and trace amount of C3b-AlexaFluor 647 was used as a positive control. As a negative control, AlexaFluor647-labeled C3b was used alone. The samples were incubated at 37 °C for 90 min, and reactions were terminated by adding reducing (DTT) sample buffer and boiling for 3 min. Proteins were separated on 10% SDS-PAGE and visualized using a Typhon scanner (GE Healthcare). Images of the gels were analyzed using ImageQuant Tl 8.1 software. The degradation of C3b in serum samples was calculated across triplicate measurements by taking the ratio of the 43-kDa degradation product and the 101-kDa α'chain in triplicate. Data were analyzed using SPSS Software version 22.0 and figures were generated using Graphpad Prism 5.03.



eFigure 1: Pedigrees of 7 AMD families in which rare variants were identified. Risk alleles are indicated in red. Affected individuals are numbered representing AMD-stage (1 early; 2 intermediate; 3 GA; 4 CNV) and age at examination is given. Individual * of family E reported kidney failure. Arrows point to family probands.

eTable 1: Number and percentage of carriers of rare variants in the case-control cohort

Variant	AMD cases (n=1831)*	%	AMD controls (n=1367)	%
CFI p.Gly119Arg	15	0.819%	1	0.073%
C9 p.Pro167Ser	61	3.332%	31	2.268%
C3 p.Lys155Gln	42	2.294%	16	1.170%
CFH p.Ser193Leu	5	0.273%	0	-
CFH p.Arg175Gln	2	0.109%	1	0.073%
CFI p.Pro553Ser	10	0.546%	2	0.146%
CFI p.Leu131Arg	4	0.218%	0	-
C9 p.Arg118Trp	3	0.164%	2	0.146%
C3 p.Arg161Trp	4	0.218%	0	-
Total*	139	7.794%	53	3.877%

* Some AMD cases carry multiple rare variants

eTable 2: Median serum levels of FH, FI, C9 and C3 in carriers and non-carriers of rare variants

	Median FH (µg/ml)	Median FI (µg/ml)	Median C9 (µg/ml)	Median C3 (µg/ml)
Cases (non-carrier)	283.0	27.2	6.6	874.0
Controls (non-carrier)	302.4	30.4	6.1	966.6
CFH p.Arg175Gln	299.4			
CFH p.Ser193Leu	266.3			
CFI p.Gly119Arg		18.2		
CFI p. Leu131Arg		16.2		
CFI p.Pro553Ser		28.4		
C9 p.Pro167Ser			10.7	
C9 p.Arg118Trp			7.4	
C3 p.Arg161Trp				943.2
C3 p.Lys155Gln				946.7

Underlined: significantly different compared to non-carrier cases and controls

eTable 3. Median C3b Degradation (43-kDA product over the alpha-chain) and quartiles.

	Median Ratio (Q1-Q3)
Cases (non-carrier)	0.724 (0.621-0.839)
Controls (non-carrier)	0.845 (0.676-0.913)
CFH p.Arg175Gln	0.387 (0.282-0.447)
CFH p.Ser193Leu	0.421 (0.315-0.454)
CFI p.Gly119Arg	0.395 (0.324-0.445)
CFI p.Leu131Arg	0.373 (0.330-0.393)
CFI p.Pro553Ser	0.532 (0.466-0.616)

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Mariann Kremlitzka*
Maartje J. Geerlings*
Sarah de Jong
Bjorn Bakker
Sara C. Nilsson
Sascha Fauser
Carel B. Hoyng
Eiko K. de Jong
Anneke I. den Hollander
Anna M. Blom
*these authors contributed equally to this study

In preparation

4

FUNCTIONAL ANALYSIS OF RARE GENETIC VARIANTS IN COMPLEMENT COMPONENT C9 IN PATIENTS WITH AGE-RELATED MACULAR DEGENERATION

ABSTRACT

Age-related macular degeneration (AMD) is a progressive disease of the central retina and the leading cause of irreversible vision loss in the western world. The involvement of abnormal complement activation in AMD has been suggested by association of genetic variants in genes encoding complement proteins with disease development. A low-frequency variant (p.Pro167Ser) in the complement component C9 (*C9*) gene was recently shown to be highly associated with AMD, however, the functional outcome of this variant remains largely unexplored. In this study, we reveal five novel rare genetic variants (p.Met45Leu, p.Phe62Ser, p.Gly126Arg, p.Thr170Ile and p.Ala529Thr) in *C9* in AMD patients, and evaluate their functional effects in vitro together with previously identified *C9* variants in AMD (p.Arg118Trp and p.Pro167Ser).

Our results demonstrate that the concentration of C9 is significantly elevated in patients' sera carrying the p.Met45Leu, p.Phe62Ser, p.Pro167Ser and p.Ala529Thr variants compared to non-carrier controls. However, no difference was observed in soluble terminal complement complex (sTCC) levels between the carrier and non-carrier groups. Comparing the polymerization tendency of the C9 variants, we demonstrate that the p.Pro167Ser mutant spontaneously aggregates. Other C9 mutant proteins fail to polymerize in the presence of zinc ions. Altered polymerization of the p.Phe62Ser and p.Pro167Ser variants associated with decreased lysis of sheep erythrocytes and ARPE-19 cells by carriers' sera. However, assays using recombinant proteins did not detect any alteration between lytic activity of the wild-type and mutant C9 proteins.

Our data suggest that the analyzed *C9* variants affect only the secretion and polymerization of C9 without influencing its classical lytic activity. Future experiments need to be performed to understand the implications of the altered polymerization of C9 in AMD pathogenesis.

INTRODUCTION

Age-related macular degeneration (AMD; MIM# 603075), a progressive eye disorder, is the major cause of irreversible vision loss in the western world.[1] AMD is a multifactorial disease in which both environmental and genetic factors contribute to pathogenesis.[2] Genetic alterations are estimated to account for 46-71% of variability in disease risk.[3] A large component of the heritability of AMD can be explained by genetic variants in the alternative pathway of the complement system. A recently published genome-wide association study[4] detected 52 (45 common and 7 rare) variants at 34 genomic regions that are independently associated with AMD. More than one third of these variants reside in or near a gene of the complement system.[4]

Activation of the complement cascade results in enzymatic-cleavage of the central component C3 into C3b and C3a. C3b is a crucial component of C3 and C5 convertases, allowing further propagation of the cascade into the terminal pathway where C5b-8 complexes incorporate into the membrane. After binding several copies of C9, the pre-formed C5b-8 complexes merge into the membrane and generate the pore-forming membrane attack complex (MAC), also known as the terminal complement complex (TCC).[5,6] Once constructed, MAC kills the target cell by inducing cell lysis, or at a reduced sublytic concentration MAC can provoke a wide array of physiologic responses ranging from apoptosis to pro-inflammatory cytokine secretion.[7-10]. MAC has been identified in the retina of AMD patients and there is a correlation between the amount of MAC deposition and the loss of RPE cells.[11,12] In addition, sublytic MAC deposition on RPE cells induces secretion of pro-inflammatory cytokines and vascular endothelial growth factor, contributing to the development of advanced AMD.[13,14]

Recently, three rare genetic variants in *C9* were reported in association with AMD,[15-17] namely p.Arg95*, p.Arg118Trp and p.Pro167Ser. The *C9* p.Pro167Ser was reported to be highly associated with AMD risk in multiple studies. [4,15,18,19] More recently, a genetic burden of *C9* variants was described in two separate AMD cohorts. The first study identified 13 rare variants (p-value $2.4x10^{-08}$)[18] and the second study (Corominas, manuscript submitted) identified 17 rare variants (p-value $5.01x10^{-03}$) in *C9*, however in both studies the burden did not remain significant after correction for multiple comparisons.

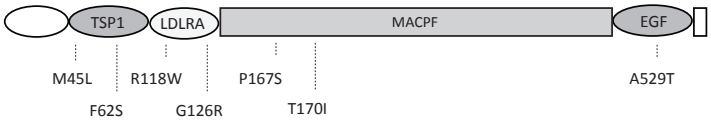
In a recent study we demonstrated that the p.Pro167Ser variant leads to increased serum concentration of the protein.[17] However, the exact functional consequences of the p.Pro167Ser variant and of other genetic alterations in *C9* remain unclear. In this study, we aimed to further elucidate the functional effects of *C9* variants *in vitro* in order to understand the role of C9 in AMD pathogenesis.

RESULTS

Genetic alterations identified in C9

Through whole-exome sequencing in 793 unrelated individuals (662 AMD cases and 131 controls) we identified five novel rare variants in *C9*, namely p.Met45Leu, p.Phe62Ser, p.Gly126Arg, p.Thr170Ile and p.Ala529Thr, in addition to previously reported *C9* p.Arg118Trp and p.Pro167Ser (**Fig. 1**). Genotyping of these seven variants in 1896 unrelated AMD cases and 1499 unrelated control individuals identified 132 rare variant carriers (**Supplementary Table 1**). In our case-control cohort, none of the rare *C9* variants were individually associated with AMD pathogenesis (**Supplementary Table 2**). However, the p.Pro167Ser variant was previously shown to be highly associated with AMD in large case-control studies,[4,15,18,19] and a burden of rare variants was recently suggested for *C9*.[18](Corominas, manuscript submitted) Therefore, we set out to identify the functional effect of these variants on the C9 protein, in order to place them into context of AMD pathogenesis.

A



B

refSNP	Amino acid	cDNA	ExAc freq (%)	PhyloP*	Grantham*	SIFT**	PolyPhen2**	CADD phred	Literature (1st report)
rs41271047	Met45Leu	133A>T	0.210	0.734	15	T	B	9.639	Novel
rs140251849	Phe62Ser	185T>C	0.010	2.187	155	T	D	25	Novel
rs147701327	Arg118Trp	352C>T	0.020	1.393	101	D	P	28	Geerlings et al., 2017
rs199939436	Gly126Arg	376G>A	0.030	3.758	125	D	D	34	Novel
rs34882957	Pro167Ser	499C>T	0.470	3.279	74	D	D	25.3	Seddon et al., 2013
NA	Thr170Ile	509C>T	NA	2.087	89	D	D	24.7	Novel
rs137891079	Ala529Thr	1585G>A	0.050	-1.191	58	T	D	0.028	Novel

Figure 1. Overview of rare genetic variants in C9 identified in AMD case-control cohort. *Higher PhyloP [range -14;6.4] and Grantham [range 0-215] scores correlate with a higher conservation. ** Sorting Intolerant from Tolerant (SIFT) and PolyPhen2 classification: (T, tolerated; B, benign; D, damaging; P, pathogenic). NA: non available

The identified variants are present in different domains of the protein: the thrombospondin type 1 (TSP1), the low-density lipoprotein receptor type A (LDLRA), the MAC/perforin (MACPF) and the epidermal growth factor-like (EGF) domains (**Fig. 1A**). All the identified variants are non-synonymous point mutations resulting in amino acid changes in the mature protein.

To study the effect of these variants in vitro, 128 serum and 95 plasma samples of the identified carriers were included in functional assays and compared to 156 sera (78 with AMD and 78

without the disease) and 155 plasma samples (77 with AMD and 78 without AMD) of age-matched non-carrier individuals (**Supplementary Table 2**).

Serum C9 and plasma terminal complement complex level of C9 carriers

To analyze whether the identified genetic variants affect secretion of the mature proteins, the concentration of C9 was measured in sera of 127 AMD patients carrying rare genetic variants in C9 and compared to 78 non-carriers with (n=78) or without (n=78) AMD. We found that the p.Met45Leu, p.Phe62Ser, p.Pro167Ser and p.Ala529Thr variants lead to a significantly increased C9 level compared to non-carrier controls (**Fig. 2A**). Sera carrying the p.Arg118Trp and p.Gly126Arg variants did not significantly differ in C9 levels between carriers and non-carriers (**Fig 2A**).

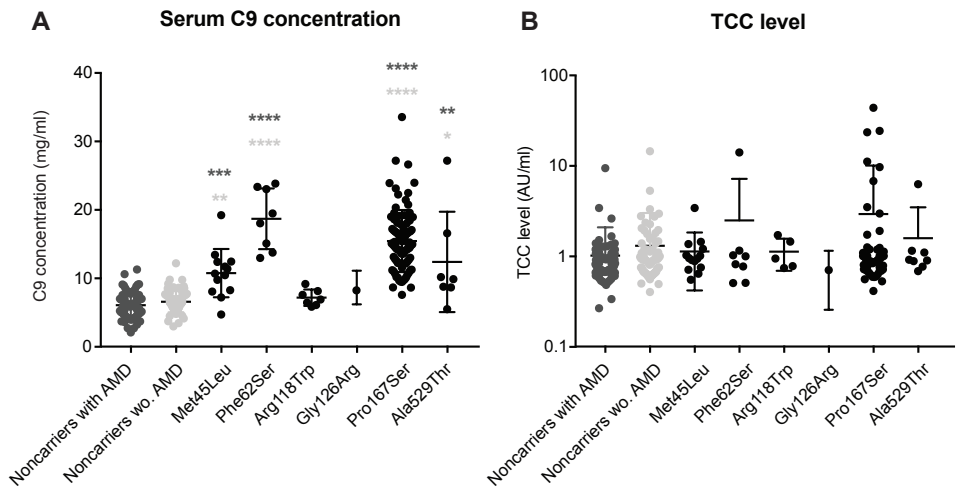


Figure 2. Measurement of serum *C9* and plasma sTCC levels in *C9* carriers. Concentrations of C9 in sera (A) and sTCC level in plasma (B) of patients carrying rare genetic variants in *C9* were measured by ELISA and compared to non-carriers with or without AMD. Differences with p<0.05 were considered statistically significant and marked with dark (carriers versus non-carriers with AMD) or with light grey asterisks (carriers versus non-carriers without AMD) (Kruskal-Wallis test with Dunn's correction, ^{ns}p>0.05, *p<0.05, **p<0.0021, *** p<0.0002, ****p <0.0001). Data are shown as median mg/ml secreted C9 or AU/ml sTCC level (the latter on log10 scale) with interquartile range of four (C9 ELISA) or three (TCC ELISA) independent experiments.

Binding of C9 to soluble C5b-8 complexes results in formation of soluble terminal complement complex (sTCC), which is a sign of ongoing complement activation. Therefore, we measured sTCC levels in patient and control sera of carriers and non-carriers of rare *C9* variants. Measurement of sTCC levels in patient and control plasma showed no differences in variant carriers compared to the control groups, even though elevated C9 concentrations are present (**Fig. 2B**)

Secretion of C9 mutants by HEK293F cells

In order to explore the impact of the identified variants on the protein function, independent of other serum components, Freestyle HEK293F cells were transfected with either wild type or mutant C9 constructs. The isolated proteins were visualized by both silver staining (data not shown) and Western blotting under non-reducing conditions (**Fig. 3A**), confirming the presence of pure, monomeric C9.

Using a similar experimental approach we compared the expression and secretion of the wild type and mutant C9 proteins. To this end, HEK293F cells were transfected with either the wild type or mutant C9 constructs, and the concentration of secreted C9 in the supernatants was analyzed by ELISA. As shown in **Figure 3B**, secretion of the p.Phe62Ser and p.Gly126Arg mutant proteins was significantly elevated compared to the wild type protein. The secretion of the p.Met45Leu, p.Arg118Trp, p.Pro167Ser, p.Thr170Ile and p.Ala529Thr mutant proteins did not significantly differ from the wild type C9 protein.

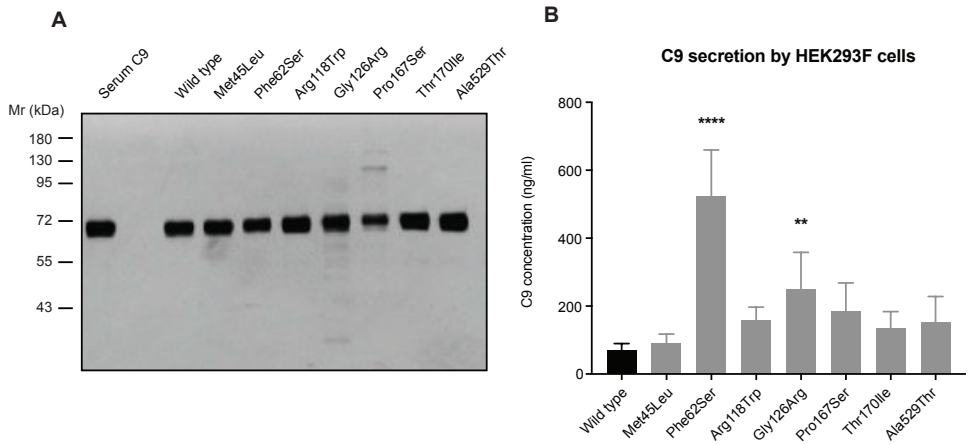


Figure 3. Expression and secretion of recombinant WT and mutant C9 variants in HEK293F cells. (A) Wild type and mutant C9 proteins were expressed in HEK293F cells and purified by Ni^{2+}-affinity chromatography and gel filtration. Purified proteins were separated under reducing conditions by 10% SDS-PAGE (0.5 mg/lane) and transferred onto PVDF membrane. The presence and purity of recombinant proteins were investigated using goat anti-human C9. Results of one representative purification out of ten is shown. (B) HEK293F cells were transiently transfected with 5 mg WT or mutant C9-pCEP4 constructs. Concentration of C9 mutant proteins in the secreted supernatants was measured by ELISA. Graphs show mean ± SD mg/ml secreted C9 of four independent experiments. Differences with $p<0.05$ were considered statistically significant (one-way ANOVA with Dunnett's multiple comparison, $^{ns}p>0.05$, $*p<0.05$, $**p<0.002$, $***p<0.0002$).

Lytic activity of recombinant C9 variants

To investigate whether the identified variants have a functional impact, the recombinant, purified wild type and mutant C9 proteins were compared in their ability to lyse erythrocytes. To this end, sensitized sheep erythrocytes were treated with C9-depleted serum which was reconstituted with the wild type or mutant C9 proteins. As demonstrated in **Figure 4A,** we observed that the lytic activity of the mutant C9 proteins did not differ from that of the wild type protein, with the exception of the p.Pro167Ser mutant protein, which showed a slight, but significant reduction in lysis of the erythrocytes.

To further investigate the effects in a more relevant model for disease development, we compared the lytic activity of the wild type and mutant C9 proteins on the retinal pigment epithelial cell line, ARPE-19. To induce MAC deposition, ARPE-19 cells were pre-incubated with a function-blocking anti-CD59 antibody and C9-depleted serum supplemented with either the wild type or mutant recombinant C9 proteins. We did not observe any differences between the wild type and mutant C9 proteins in their cytotoxic activity on ARPE-19 cells (**Fig. 4B**).

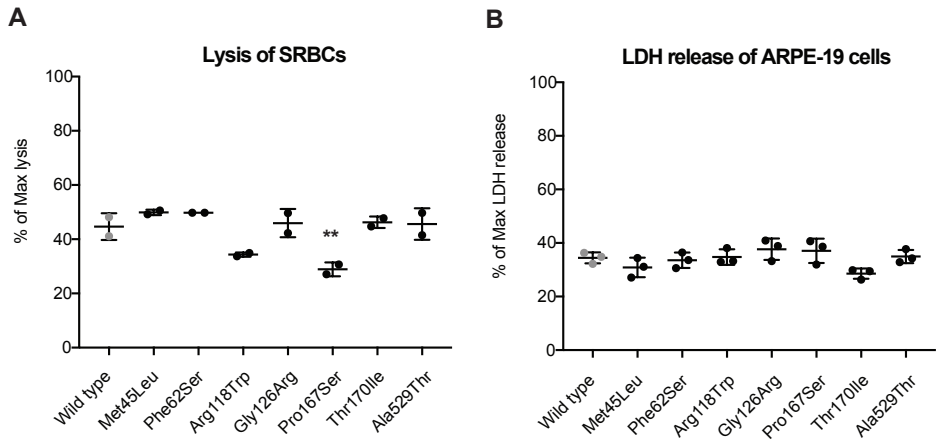


Figure 4. Lytic activity of recombinant C9 variants. Sheep red blood cells (SRBCs)(A) or ARPE-19 cells (B) were treated with C9-depleted serum supplemented with the recombinant WT or mutant C9 proteins. (A) Lysis of SRBCs was analyzed via measurement of released hemoglobin at 405 nm. Data are expressed as median with interquartile range of water-induced maximum lysis and are results of three independent experiments carried out in duplicate. (B) MAC-induced cytotoxicity was measured via lactate dehydrogenase (LDH) release from ARPE-19 cells. Data expressed are mean ± SD of lysis buffer-induced maximum lysis and are results of three independent experiments measured in duplicate. Differences with $p<0.05$ were considered statistically significant (one-way ANOVA with Dunnett's multiple comparison, $^{ns}p>0.05$, $^{**}p<0.0021$).

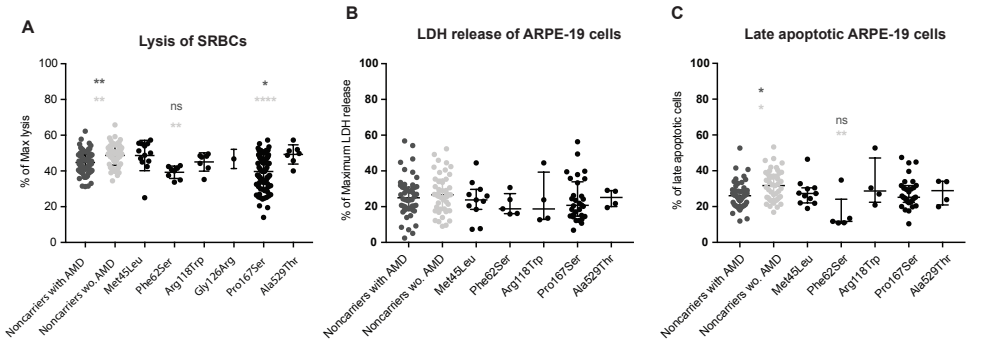


Figure 5. C9-dependent lytic activity of sera of carriers and non-carriers of rare genetic variants in *C9*. Lytic activity of sera was measured by incubation of SRBCs (A) or ARPE-19 cells (B and C) with C9-depleted serum supplemented with EDTA-GVB diluted sera of carriers and non-carriers. (A) Lysis of SRBCs was analyzed via measurement of released hemoglobin at 405 nm. Data are expressed as median with interquartile range of water-induced maximum lysis and are results of four independent experiments. (B and C) MAC-induced cytotoxicity of ARPE-19 cells was measured via LDH release (B) and by analysis of Annexin V+ZombieAqua+ (late apoptotic) cells by flow cytometry (C). Data are expressed as median with interquartile range of lysis buffer-induced maximum LDH release (B) and % of late apoptotic cells (C) of two independent experiments measured in duplicate. Differences with $p<0.05$ were considered statistically significant and marked with dark (carriers versus non-carriers with AMD) or with light grey asterisks (carriers versus non-carriers without AMD) (Kruskal-Wallis test with Dunn's multiple comparison, $^{ns}p>0.05$, $*p<0.05$, $**p<0.0021$, $****p<0.0001$).

Lytic activity of sera of C9 carriers

A modified hemolytic assay was designed to measure the C9-dependent lytic activity independent of other complement components. Sensitized erythrocytes or ARPE-19 cells were incubated with C9-depleted serum in DGVB++ (dextrose gelatin veronal buffer), which allows complement activation and deposition of C5b-8 complexes on the cell membrane. Thereafter the cells were incubated with sera of carriers or non-carrier controls diluted in EDTA-GVB (ethylenediaminetetraacetic acid - gelatin veronal buffer), which blocks complement activation and novel C5b-8 complex formation, but allows integration of C9 in the pre-formed C5b-8 complexes and induces lysis dependent on both the protein function and C9 serum concentration.

In spite of the significantly increased C9 concentration in sera of the p.Met45Leu, p.Phe62Ser, p.Pro167Ser and p.Ala529Thr carriers, the elevated C9 level did not cause increased lysis of erythrocytes (**Fig. 5A**) or ARPE-19 cells (**Fig. 5B and 5C**). On the contrary, we observed a slight but significant decrease in lytic activity for *C9* p.Phe62Ser on both erythrocytes and ARPE-19 cells compared to non-carrier controls without AMD. Furthermore, the *C9* p.Pro167Ser variant showed decrease in lytic activity on erythrocytes compared to non-carriers with or without AMD (**Fig. 5A and Fig. 5C**).

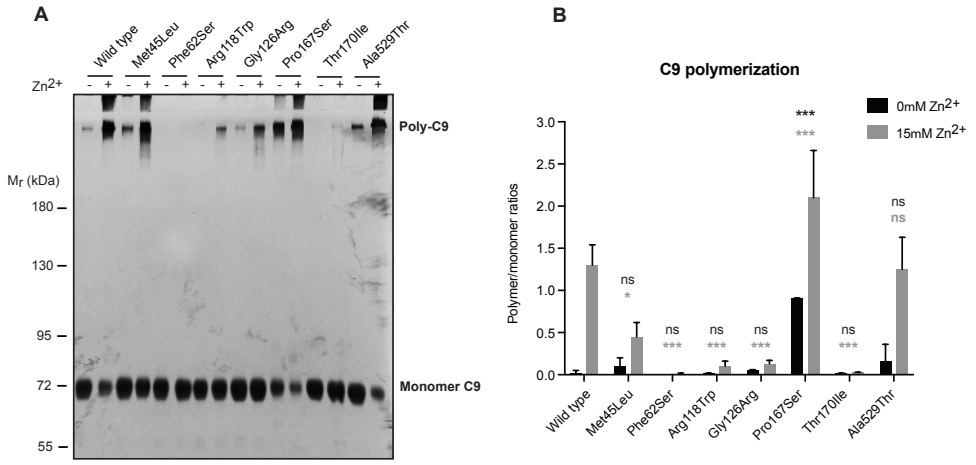


Figure 6. Polymerization of WT and mutant C9 proteins in the presence or absence of Zn^{2+} ions. Recombinant WT or mutant C9 (5 mM) were allowed to polymerize in the presence or absence of 15 mM Zn^{2+} ions at 37°C for 4h. The proteins were separated by electrophoresis on a 5-10% polyacrylamide gradient gel and polymers were visualized by silver staining. (A) One representative experiment of three is shown. (B) Densitometric analysis of polymer and monomer ratios of WT and mutant C9 proteins were calculated by ImageLab software. Results shown are the densitometric ratios of polymer and monomer C9 bands and illustrated as median with interquartile range of three independent experiments. Differences between WT and mutant C9 proteins with $p<0.05$ were considered statistically significant and marked with dark (spontaneous polymerization) or with light grey asterisks (Zn^{2+}-induced polymerization) (one-way ANOVA with Dunnett's multiple comparison, $^{ns}p>0.05$, $^{**}p<0.021$, $^{****}p<0.0001$).

Polymerization of recombinant C9 mutant proteins

During purification of recombinant C9 proteins we observed that the p.Pro167Ser mutant protein tends to aggregate and form high molecular weight aggregates (**Fig. 3A**). To evaluate the polymerization tendency of the C9 mutant proteins, purified C9 was incubated in the presence or absence of zinc ions, which accelerate C9 polymerization and are used as dietary supplement to reduce AMD progression[20]. We observed that the p.Pro167Ser mutant protein spontaneously aggregates (**Fig. 6**). In the absence of zinc, the other mutant proteins polymerized normally. However, the p.Met45Leu, p.Phe62Ser, p.Arg118Trp, p.Gly126Arg and p.Thr170Ile mutant proteins showed impaired polymerization in the presence of zinc in contrast to the p.Pro167Ser mutant protein, which showed higher polymerization than the wild type protein. Polymerization ability of the p.Ala529Thr mutant protein was unaltered.

DISCUSSION

In this study, we studied the functional effect of the p.Pro167Ser variant in *C9*, which was in multiple recent studies found to be highly associated with AMD.[4,15,18,19] In addition, a burden of rare genetic variants in *C9* was recently suggested by two independent studies.[18](Corominas, manuscript submitted) In this study we identified five novel, non-synonymous rare genetic variants in *C9* (p.Met45Leu, p.Phe62Ser, p.Gly126Arg, p.Thr170Ile and p.Ala529Thr), in addition to the previously identified *C9* p.Arg118Trp[17] and p.Pro167Ser variants.[15] The identified C9 variants (except for *C9* p.Met45Leu variant) were more prevalent in individuals affected by AMD compared to control individuals (**Suppl. Table 2**). However, due to the limited number of individuals analyzed in the current study, in combination with the low allele frequency of the C9 variants, the association to AMD was statistically inconclusive. Functional characterization of the identified variants on the C9 protein may provide insight in the role of C9 in AMD pathogenesis.

For C9, only three genetic variants have previously been described in AMD. A non-sense variant (p.Arg95*) leads to complete or partial C9 deficiency.[21] This variant is protective for AMD, conferring a 4.7-fold reduction in disease risk, and is correlated to decreased vascular endothelial growth factor levels.[16] The p.Arg118Trp C9 variant was identified with a relatively low allelic odds-ratio (1.12) and did not alter the C9 level in carriers compared to non-carriers.[17] *C9* p.Pro167Ser confers risk for AMD[4,15,17-19] and results in elevated serum C9 levels in carriers compared to non-carriers.[17]

We did not detect any significant alteration in systemic complement activation levels, measured in the form of plasma sTCC between the carrier, non-carrier, affected and unaffected individuals in serum (**Fig. 2B**). This is in accordance with previous studies, reporting no significant difference in systemic sTCC level between AMD patients and non-carrier control groups.[22-24] Increased MAC (soluble C5b-9 MAC and immunohistochemistry staining) was previously observed in Bruch's membrane and the choriocapillaris of human eyes, gradually increasing with age. The elevated MAC was especially evident in eyes affected by AMD, even more so in carriers of the *CFH* risk allele.[11,12] These findings suggest that changes in MAC are associated with AMD but can only be detected locally.

We found that carriers of the p.Met45Leu, p.Phe62Ser, p.Pro167Ser and p.Ala529Thr variants have increased serum C9 levels compared to non-carrier controls (**Fig. 2A**). However, carriers of the p.Arg118Trp and p.Gly126Arg variants did not have altered C9 serum concentrations compared to sera of non-carrier controls. Unfortunately, no serum was available for p.Thr170Ile. These data confirm our earlier results reporting normal C9 levels in sera carrying the C9 p.Arg118Trp variant and elevated C9 levels in carriers of the p.Pro167Ser variant.[17]

Interestingly, in HEK293F cells secretion of the recombinant C9 proteins was higher for the C9 p.Phe62Ser and p.Gly126Arg mutant proteins (**Fig. 3B**), but not for the p.Met45Leu, p.Pro167Ser and p.Ala529Thr mutant proteins. The discrepancy between results of C9 ELISA from serum and cell culture supernatants may be due to the fact that *in vivo* C9 is secreted by various cell types which express the protein at distinct level in contrast to our in vitro system, where C9 was produced by a single cell type.

Despite the significantly elevated C9 levels in carriers of the p.Met45Leu, p.Phe62Ser, p.Pro167Ser and p.Ala529Thr variants (**Fig. 2A**), we did not detect an increase in C9-dependent lytic activity of patients´ sera carrying these rare genetic variants in *C9* (**Fig. 4**). More surprisingly, the sera of carriers of the p.Phe62Ser and p.Pro167Ser C9 variants caused decreased lysis of sheep erythrocytes or ARPE 19 cells. The lytic activity of the sera of carriers of p.Met45Leu and p.Ala529Thr C9 variants was not altered, despite increased serum levels (**Fig 5A and Fig. 5C**). One would assume that an increased C9 concentration would result in enhanced MAC formation and thus, increased killing of the target cells. Nevertheless, it should be considered that the stochiometry of C5b-8 and C9 within MAC is strictly regulated. One C5b-8 complex can bind only a limited number of C9 molecules [25], therefore, even when more C9 is available, it does not necessarily lead to more C5b-9 complex and hence, enhanced pore formation and lysis.

Hemolytic assays performed with the recombinant C9 proteins purified on a nickel column (containing both monomeric and polymeric proteins) displayed a significantly reduced lytic activity of the C9 p.Phe62Ser and p.Pro167Ser mutant proteins (data not shown). We confirmed these observations using serum samples (**Fig. 5A**). To exclude the functionally inactive polymers from our analysis, the recombinant C9 proteins were further purified by gel filtration (**Fig. 3A**). Notably, applying the pure, monomer forms of C9, we did not detect any differences in lytic activity of the wild type and mutant proteins (**Fig. 4**), suggesting that the genetic alterations do not alter the classical, lytic function of C9.

C9 has a tendency to polymerize, leading to formation of poly(C9) and a rapid loss of hemolytic activity[26]. Although we could not detect the presence of poly(C9) in sera of the p.Pro167Ser carriers, our in vitro data suggest that the C9 p.Pro167Ser mutant protein has an increased tendency to aggregate. Formation of polymers of C9 p.Pro167Ser mutant proteins could already be observed during expression and purification of the recombinant protein. In contrast, the C9 p.Phe62Ser mutant protein had a decreased capacity to polymerize in the presence of zinc ions (**Fig. 6**). Despite the almost complete lack of zinc-induced polymerization, the hemolytic activity of the C9 p.Phe62Ser mutant protein was only slightly reduced (**Fig. 5**). This suggests that the C9 p.Phe62Ser variant only slightly impairs the capacity of C9 to bind C5b-8.

In our experiments, zinc was used to induce polymerization since formation of poly(C9) is accelerated by the metal ion.[27] However, zinc is also used in dietary supplements to reduce AMD progression.[20,28] The zinc concentrations we used are within the same range as recommended for supplementation. Zinc treated C9 mutant (p.Pro167Ser) polymerizes and is thus not able to bind to C5b-8 and unable to cause lysis. However, the polymerization tendency of almost all other C9 mutants is reduced in the presence of zinc compared to wildtype C9. This suggests that zinc supplementation may be ineffective at reducing MAC associated lysis in patients carrying the *C9* mutations.

Previously, C9 polymerization was reported to occur rapidly in the presence of C5b-8 complexes, while spontaneous poly(C9) formation takes several days at 37°C [26]. The rapid polymerization of the C9 p.Pro167Ser mutant protein even in the absence of C5b-8 complexes or zinc ions (**Fig. 6**) indicate that functionally inactive aggregates can already be formed during the assay, leading to reduced lysis of the target cells even when applying the protein in monomer form (**Fig. 4A**). In contrary, polymerization of the C9 p.Phe62Ser mutant protein was only observed in the presence of C5b-8 complexes (**Fig.4**) or during longer incubation time, for example during production and purification of the protein. This may explain the discrepancy between decreased lytic activity of patients' sera carrying the p.Phe62Ser C9 variant (**Fig. 5**) and of mixed polymer-monomer solution of purified C9 p.Phe62Ser mutant protein (data not shown) in contrast to normal lytic activity of the purified, monomer protein (**Fig. 4**). The decreased lysis we observed could be explained by altered polymerization tendency of C9 p.Phe62Ser and by spontaneous aggregation of the p. P167S mutant protein (**Fig. 5A; Fig 6**).

The *in vitro* assays showed altered polymerization and decreased lytic activity of C9 p.Phe62Ser and p.Pro167Ser mutant proteins, while this was not seen for the other C9 mutant proteins. This may be explained by the localization of the variants in the mature protein (**Fig. 1**): the TSP1 and MACPF domains have been reported as main drivers of C9 polymerization during MAC formation.[29] Thus, the p.Phe62Ser variant and in particular the p.Pro167Ser variant (due to the substitution of a proline residue) may alter the structure of C9 in a way that results in impaired pore formation and lysis.

Taken together, we have shown that several identified C9 variants, including the p.Pro167Ser variant highly associated with AMD, affect the serum level and polymerization of C9 without influencing its classical lytic activity. These results, alongside with normal sTCC level of the carriers' plasma and the lack of correlation between C9 concentration and lytic activity of patients' sera, suggest that the variants influence disease pathology locally in the eye but not by increased lytic activity. Future experiments are needed to understand the implications of the altered polymerization on AMD pathogenesis. We hypothesize that accumulation of aggregated

protein within the cell induces (endoplasmatic reticulum) stress, which could eventually lead to apoptosis of the cells.

MATERIALS AND METHODS

Genetic analysis and patient selection

We performed whole-exome sequencing for 793 unrelated individuals (662 cases and 131 controls), as described in detail previously[17]. Filtering steps were implemented to uncover the coding non-synonymous variants of *C9* (NM_001737.3). Frequency filters, using public database ExAC [30] were implemented to ensure selection of rare variants (minor allele frequency <1%). We identified seven unique rare variants in *C9* which were screened in an additional cohort consisting of 1234 unrelated case and 1368 unrelated control individuals using custom-made competitive allele-specific PCR assays according to manufacturers' recommendations (KASP SNP Genotyping System, LGC). Furthermore, family members of 8 different families (n=17) of which the proband carried a *C9* variant were screened using Sanger sequencing.

All individuals included in the study underwent clinical evaluation and were graded for 'AMD' or 'no AMD' according to the Cologne Image Reading Center protocol. Control individuals without AMD were 60 years or older [31].

Collection of serum and plasma samples

Serum and plasma samples were obtained by a standard coagulation/centrifugation protocol and frozen at –80°C within 1 hour after collection. Genomic DNA was isolated from peripheral blood samples according to standard procedures. This study was approved by local ethics committees on Research Involving Human Subjects and met the criteria of the Declaration of Helsinki.

To analyze the functional effect of rare genetic variants in C9, 128 serum and 95 plasma samples of 132 carriers were collected. Furthermore, we obtained serum (n=156) and plasma (n=155) of 157 individuals that did not carry a rare genetic variant in *C9* (**Supplementary Table 2**).

Cell lines

Freestyle HEK293F cells (Invitrogen) were cultured in Freestyle Expression medium (Invitrogen) according to the manufacturer's instructions. Cells were passaged every third day and transfected at passage 10. The retinal pigment epithelial cell line ARPE-19 (ATCC) was cultured in DME/F12 medium (Hyclone) supplemented with 10% heat-inactivated FCS (Hyclone). Cells used for functional assays were between passage 10 and 20. The cells were *mycoplasma* free and tested regularly with the VenorGEM Classic kit (Minerva Biolabs).

ELISA for measurement of C9 concentration

C9 concentrations in serum samples or supernatants of HEK293F cells secreting C9 recombinant proteins were determined by ELISA. Maxisorp microtiter plates (96-well, Thermo Scientific) were coated with 1 µg/mL mouse anti-human C9 (Hycult Biotech) in 50 mM sodium carbonate (pH 9.6) overnight, at 4 °C. Between each of the following steps, the plates were washed four times with Immunowash (50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 2 mM $CaCl_2$, 0.1% Tween 20). After coating, plates were blocked in Quench solution (Immunowash supplemented with 3% fish gelatin, Nordic) for 1h at room temperature (RT) and incubated with serum samples (diluted 1:200 in Quench) or with supernatants of HEK293F cells for 1h at RT. As standard, serum-purified C9 (prepared in our lab) was applied. After incubation, bound proteins were detected using goat anti-human C9 (1:4000 in Quench, Complement Technologies) followed by HRP-conjugated rabbit anti-goat Ig (1:2000 in Quench, Dako). As substrate, 1,2-phenylenediamine dihydrochloride (OPD, Dako) was used and absorbance at 490 nm was measured using a Cary50 MPR microplate reader (Varian).

ELISA for measurement of sTCC concentration

Maxisorp immunoplates (96-well) were coated with 0.5 µg/mL mouse anti-human TCC (aE11, Hycult) in PBS (pH7) (GE Healthcare) at RT, overnight. Between each of the following steps, plates were washed four times with PBST (PBS + 0.2% Tween 20). After coating, the plates were incubated with plasma samples diluted 5X in AG buffer (PBS + 0.02% NaN3 + 0.2% Tween 20 + 0.02 M Na_2EDTA) for 1h at RT. As standard, zymosan activated serum (ZAS-93, 1000AU/ml, Hycult) was used. Plasma sTCC was detected using biotinylated mouse anti-human C6 (1:2000 in PBST, Quidel) followed by HRP-conjugated streptavidin (1:1000 in PBST, R&D Sytems). As chromogen, OPD was used.

C9 cDNA clones for recombinant proteins

To determine if the genetic variants affect the function of the protein, both the wild type (WT) and mutant C9 proteins were expressed *in vitro*. To this end, full-length cDNA encoding human *C9* with an N-terminal His-tag was purchased from Invitrogen in pMA-T vector. The identified variants in *C9*, *i.e.* p.Met45Leu, p.Phe62Ser, p.Arg118Trp, p.Gly126Arg, p.Pro167Ser, p.Thr170Ile and p.Ala529Thr were introduced using the QuikChange site-directed mutagenesis kit (Agilent Technologies), according to manufacturer's instructions. The primers used are listed in Supplementary Table 1. The variants were confirmed by automated Sanger DNA sequencing (GATC Biotec). Wild type and mutant C9 cDNA sequences were then subcloned into the eukaryotic pCEP4 expression vector (Invitrogen), suitable for transfection of HEK293F cells.

Expression and purification of recombinant C9 proteins

HEK293F cells were transiently transfected with the wild type or mutant C9-pCEP4 constructs using FreeStyle Max Reagent according to manufacturer's instructions (Invitrogen). Secreted

supernatants were collected after 2, 4, 6, 8 days, pooled and stored at -20°C. Recombinant wild type and mutant C9 proteins were isolated from the collected media using a column of Ni-NTA Superflow resin (Qiagen) equilibrated with 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH8 (binding buffer). After extensive washing with binding buffer, bound proteins were eluted with 50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole, pH8 (elution buffer). The presence of C9 in the eluted fractions was verified by 7.5% SDS-PAGE and silver staining. To separate monomer and polymer C9 proteins by gel filtration, C9-containing fractions of Ni^{2+}-affinity chromatography were pooled, concentrated using an Amicon Filter unit with 50kDa cut-off (Millipore) and loaded onto Sephacryl-100 column (GE healthcare) to separate monomer and polymer C9 proteins. All preparative works were done at 4°C. The purity of monomer wild type and mutant C9 proteins was analyzed by SDS-PAGE and Western blot.

SDS-PAGE and Western blot

The purified recombinant C9 proteins were separated by gel electrophoresis under reducing (25 mM DTT) conditions and transferred to a PVDF membrane using semi-dry blotting apparatus (BioRad). The membranes were blocked with Quench solution and C9 was visualized using a polyclonal, goat anti-human C9 antibody from Complement Technologies (1:20.000 dilution in Quench) followed by incubation with HRP-conjugated rabbit anti-goat Ig (diluted 1:10.000 in Quench). Bound antibodies were visualized by the enhanced chemiluminescence (ECL) method (Millipore) and analyzed with the ImageLab software (BioRad).

Lysis of erythrocytes by recombinant wild type and mutant C9 proteins

Sheep erythrocytes (Håtunalab) were washed with dextrose gelatin veronal (DGVB++) buffer (2.5 mM veronal buffer, pH 7.3, 72 mM NaCl, 140 mM glucose, 0.1% gelatin, 1 mM $MgCl_2$, and 0.15 mM $CaCl_2$), pelleted and incubated with 1 ml of DGVB++ containing amboceptor (Behring) diluted 1:1000 for 20 min at 37°C. Sensitized erythrocytes were washed three times, pelleted and resuspended in DGVB++ to obtain a cell suspension of which 10 µl lysed by 90 µl of water gives 1.2-1.4 absorbance at 405 nm. The suspension (10 µl) was incubated with 50µl of 2% C9-depleted serum (Complement Technologies) reconstituted with 50 ng/ml wild type or mutant C9 proteins (preliminary titrated concentration of recombinant wild type C9 causing lysis of 50% of erythrocytes). After incubation for 30 min at 37°C, cells were overlayed with another 50 µl of DGVB++ and centrifuged. Released hemoglobin levels of the collected supernatants (80 µl) were measured at 405 nm using Cary50 MPR microplate reader.

Lysis of erythrocytes by sera of C9 carriers and non-carrier controls

To analyse C9-dependent lysis of erythrocytes by sera of carriers and non-carriers, erythrocytes were prepared and sensitized as described above. To induce deposition of C5b-8 complexes, 10 µl of the erythrocyte suspension was incubated with 50 µl of 2% C9-depleted serum (Complement Technologies) diluted in DGVB++ at 37°C for 30 min, shaking at 650 rpm. After incubation, cells

were washed three times with 40 mM EDTA-GVB buffer (2.5 mM veronal buffer, pH 7.3, 72 mM NaCl, 140 mM glucose and 40mM EDTA) and MAC assembly was induced by serum samples, diluted 1:2000 in 40 mM EDTA-GVB (the buffer blocks complement activation and thus prevents formation of novel C5b-8/9 complexes from the applied sera). After incubation for 30 min at 37°C, cells were overlayed with another 50 µl of 40mM EDTA-GVB and centrifuged. Released hemoglobin level of the collected supernatants (80 µl) was measured at 405 nm using a Cary50 MPR microplate reader.

Treatment of ARPE-19 cells using recombinant wildtype and mutant C9 proteins

ARPE-19 cells were plated at 10^6 cells/ml concentration on 96-well plates (Nunc) in DME/F12 medium supplemented with 10% FCS. After 1 day, cells were washed and medium was changed to DME/F12 without FCS. The next day, cells were centrifuged and treated with 3 µg/well function-blocking anti-CD59 antibody (IBGRL) for 45 min at 4°C to enhance MAC formation. After incubation, cells were treated with 10% C9-depleted serum reconstituted with 10 µg/ml of purified wild type or mutant C9 proteins in DGVB++ for 2h at 37°C, shaking at 150 rpm. After 2h, cells were centrifuged at 1500 g for 3min, and supernatants were collected to measure lactate dehydrogenase (LDH) release using the Pierce LDH cytotoxicity detection kit (ThermoScientific).

Treatment of ARPE-19 cells by sera of C9 carriers and non-carrier controls

ARPE-19 cells were plated and primed for MAC deposition as described above. To induce C5b-8 deposition, anti-CD59 sensitized cell were treated with 20% C9-depleted serum in DGVB++ for 45 min at 37°C, shaking at 150 rpm. After incubation, cells were washed three times in EDTA-GVB and treated with diluted serum samples. Serum samples were diluted 2.5 times in 40 mM EDTA-GVB. After 2h, supernatants were collected to measure LDH release and cells were analyzed for MAC deposition by flow cytometry.

Lactate-dehydrogenase (LDH) assay

LDH release was measured in 80µl volume using the Pierce LDH cytotoxicity detection kit (ThermoScientific) according to the manufacturer's instruction. LDH release was calculated by subtracting the background LDH activity of individual sera and medium treated cells (spontaneous LDH release control) from the sera/purified C9-treated cell culture supernatants and divided by the total LDH activity (maximum LDH Release induced by 1X lysis buffer), multiplied by 100.

Flow cytometry

To detect MAC deposition and viability of ARPE-19 cells after induction of MAC assembly, cells were washed twice in PBS and incubated with a neoantigen specific rabbit anti-human C5b-9 antibody (Complement Technologies, 1:400) for 30min at 4 °C. The cells were washed twice in

binding buffer (10mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$) and incubated with AlexaFluor647-conjugated goat anti-rabbit Ab (ThermoScientific, 1:2000), Annexin V-FITC (Immunotools, 2µl/sample) and ZombieAqua fixable viability dye (BioLegend, 1:2000) for 30 min at RT in the dark. The stained cells were washed twice and analyzed by CytoFlex flow cytometer (Beckman Coulter). Data were analysed with FlowJo software (Tree Star) and expressed as geometric mean fluorescence intensity (gMFI) of C5b-9 signal or % of ZombieAqua and Annexin V double positive, late apoptotic cells.

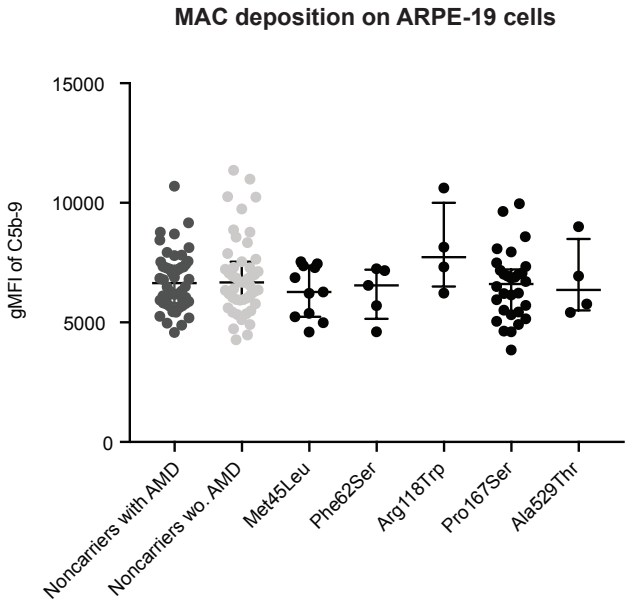
C9 polymerization

Purified C9 protein (5 µM) was allowed to polymerize in TBS in the presence or absence of 15 µM Zn^{2+} ions at 37°C for 4h, shaking at 350 rpm. After incubation, samples were separated by 5-10% gradient gel electrophoresis and visualized by silver staining.

Statistical analysis

Results investigating the relationship between serum C9 concentration, plasma sTCC level and lytic activity of serum samples and the carrier status were analyzed using the Kruskal-Wallis test with Dunn's multiple comparison. Data comparing the functional activity and polymerization of recombinant wild type and mutant C9 proteins were analysed by one-way ANOVA with Dunnett's multiple comparison. Data were analyzed and figures prepared using Prism Software version 7.

SUPPLEMENTARY INFORMATION



Supplementary Figure 1: Lytic MAC deposition on ARPE-19 cells. ARPE-19 cells were incubated with C9-depleted serum supplemented with EDTA-GVB diluted sera of carriers of *C9* variants and non-carrier controls. MAC assembly on the cell surface was analyzed using a neoantigen specific anti-C5b-9 antibody by flow cytometry. Data expressed are median with interquartile range of geometric mean fluorescence intensity (gMFI) of two independent experiments measured in duplicate. Differences with $p<0.05$ were considered statistically significant (Kruskal-Wallis test with Dunn's multiple comparison, $^{ns}p>0.05$).

Supplementary Table 1 Number of patients included in this study.

	AMD (n)	Control (n)	Total (n)	Carriers identified (n)
Whole exome sequencing	662	131	793	43
Allele-specific PCR assay	1234	1368	2602	84
Sanger-sequencing	13	4	17	13*
Serum collected	160	124	284	128
Plasma collected	139	111	250	95

*excluding 8 probands carrying a rare variant in the case-control analysis

Supplementary Table 2 Case-control frequency of rare genetic variants in *C9* and number of sera and plasma included in functional assays.

	Heterozygous carriers		Noncarriers		Allelic odds-ratio	Fisher two-tailed p value	Analyzed	
	Control (n)	AMD (n)	Control (n)	AMD (n)			Serum (n)	Plasma (n)
Met45Leu	10	9	684	654	1.554	1.000	13	14
Phe62Ser	2	5	1381	1778	1.943	0.478	8	8
Arg118Trp	2	3	1466	1828	1.203	1.000	7	5
Gly126Arg	0	1	685	662	ND	1.000	1	1
Pro167Ser	33	60	1484	1843	1.606	0.092	91	59
Thr170Ile	0	1	685	662	ND	0.491	0	0
Ala529Thr	2	4	1457	1854	1.572	0.701	7	8
Noncarriers with AMD							78	77
Noncarriers without AMD							78	78

ND: not determinable

Supplementary Table 3 Forward primers used in site-directed mutagenesis to generate C9 variants.

Mutation	Forward primers (5' to 3')*
M45L	CACACATAGACTGCAGA**T**TGAGCCCCTGGAGTGAATG
F62S	CTTGTCTCAGACAAATGT**C**TCGTTCAAGAAGCATTG
R118W	CATAAAGATGCGACT**T**TGGTGTAATGGTGACAATG
G126R	GGTGACAATGACTGC**A**GAGACTTTTCAGATGAGG
P167S	CATTTTAGGGATGGAT**T**CCCTAAGCACACCTTTTG
T170I	GGGATGGATCCCCTAAGCA**T**ACCTTTTGACAATGAGTTC
A529T	GATGGAAAGTGTTTGTGT**A**CCTGCCCATTCAAATTTGAG

* Mutations are marked in boldface and underlined.

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Maartje J. Geerlings*
Eveline Kersten*
Joannes M.M. Groenewoud
Lars G. Fritsche
Carel B. Hoyng
Eiko K. de Jong
Anneke I. den Hollander
*these authors contributed equally to this study

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GEOGRAPHICAL DISTRIBUTION OF RARE VARIANTS WHICH ARE ASSOCIATED WITH AGE-RELATED MACULAR DEGENERATION

ABSTRACT

Purpose: A recent genome-wide association study by the International Age-related Macular Degeneration Genomics Consortium (IAMDGC) identified seven rare variants that are individually associated with age-related macular degeneration (AMD), the most common cause of vision loss in elderly. In literature, several of these rare variants have been reported with different frequencies and odds ratios across populations of Europe and North America. Here, we aim to describe the representation of these seven AMD-associated rare variants in different geographical regions based on 24 AMD studies.

Methods: We explored the occurrence of seven rare variants independently associated with AMD, namely (*CFH* rs121913059 (p.Arg1210Cys), *CFI* rs141853578 (p.Gly119Arg), *C3* rs147859257 (p.Lys155Gln), and *C9* rs34882957 (p.Pro167Ser) and three non-coding variants in or near the *CFH* gene (rs148553336, rs35292876, rs191281603), in 24 AMD case-control studies. We studied the difference in distribution, interaction and effect size for each of the rare variants based on the minor allele frequency within the different geographical regions.

Results: We demonstrate that two rare AMD-associated variants in the *CFH* gene (rs121913059 [p.Arg1210Cys] and rs35292876) deviate in frequency among different geographical regions (p=0.004 and p=0.001, respectively). The risk estimates of each of the seven rare variants were comparable across the geographical regions.

Conclusion: Our results emphasize the importance of identifying population-specific rare variants, for example by performing sequencing studies in case-control studies of various populations, because their identification may have implications for diagnostic screening and personalized treatment.

INTRODUCTION

Genetic diversity is observed among populations of different ancestries. Allele frequencies can exhibit large diversity among populations due to forces like genetic drift and natural selection. While most common variants are shared worldwide, rare variants (minor allele frequency [MAF] <1%) have the tendency to cluster in specific populations. Particularly population-specific rare variants tend to have a strong functional effect[1].

In age-related macular degeneration (AMD), large variability in rare variant frequency has been reported in case-control studies of various populations, for instance for variant rs121913059 (p.Arg1210Cys) in complement factor H (*CFH*). *CFH* rs121913059 was first reported in a case-control study from the United States[2]. While some studies could replicate the finding[3-5], other Caucasian studies [6-9] and Asian studies [10,11] were unable to replicate its strong association (**Table 1**). Another example, variant rs141853578 (p.Gly119Arg) in complement factor I (*CFI*) first reported in a European cohort[7], was screened both in a British[12] and American[13] cohort (OR = 22.2; 8.5 and 2.6, respectively). However, while the variant was associated with AMD, its risk effect size was much weaker when compared to the first report.

Table 1. Minor allele frequencies of the *CFH* rs121913059 (p.Arg1210Cys) variant among different geographical regions reported in literature.

	Source	Carriers (n)	Total Cases (n)	Total Controls (n)	MAF Cases (%)‡	MAF Controls (%)‡	Odds-Ratio	P-value
World	Fritsche2016 [3]	108	16144	17832	0.319	0.014	20.3	8.9×10^{-24}
Eastern USA	Raychaudhuri2011 [2#]	34	2414	1120	0.684	0.045	NA	8.0×10^{-5}
	Zhan2013 [5]	24	2268	2268	0.507	0.022	23.1	2.9×10^{-6}
European	Helgason2013 [8]	0	1143	51435	0	0	NA	NA
	Saksens2016 [9*]	0	1589	1386	0	0	NA	NA
	Recalde2016 [4]	5	259	330	0.965	0	NA	NA
Asian	Shen2012 [11]	0	258	426	0	0	NA	NA
	Miyake2015 [10]	1	1364	1208	0.037	0	NA	NA

Additional publications from: the Boston study[#13,15] and EUGENDA study[*6,7]. ‡ major allele C, minor allele T. NA = Not available or not reported.

In a recent genome-wide association study of the International Age-related Macular Degeneration Genomics Consortium (IAMDGC)[3] seven rare variants were observed to independently confer risk for AMD. All seven rare variants are localized in or near genes encoding components of the complement system, namely *CFH, CFI*, and complement components 3 and 9 (*C3* and *C9*).

The difference in association for rare variants among different AMD case-control studies may reflect the difference in distribution of such rare alleles across geographical regions. This observation raises the question if these variants identified by the IAMDGC are represented in all case-control studies or whether the association is driven by one or more studies from a specific geographical region. Therefore, we sought to evaluate the representation of these seven AMD-associated rare variants in 24 AMD case-control studies of different geographical regions.

MATERIALS AND METHODS

Data for this study were provided by the IAMDGC. The genotypes are in part available via dbGaP under accession number phs001039.v1.p1. The original dataset contained data from 40,633 individuals of European ancestry as described by Fritsche et al.[3] For analyses of the current study, participants from the Utah case-control study were excluded due to their mixed regions of origin. Also, the Jerusalem case-control study was excluded due to its small sample size compared to the other geographical regions. Final analyses were performed on 39,582 participants deriving from 24 of 26 studies[3]. The included studies were grouped in five geographical regions: eastern USA, western Europe, Britain, western USA and Australia (**S1 Table**). Data were collected by all study groups in accordance with the tenets of the Declaration of Helsinki; participants provided informed consent and study protocols were approved by local ethical committees[3].

The MAF in each region was calculated and compared independently of AMD status. For comparison of effect sizes and interaction analyses, individuals were assigned "AMD" when exhibiting signs of (1) advanced AMD defined as geographic atrophy and/or choroidal neovascularization in at least one eye, or (2) non-advanced AMD defined as pigmentary changes in the macula and/or more than five macular drusen with a diameter ≥63 μm. Individuals without any reported signs of AMD were assigned "No AMD".

Genotype data of seven rare genetic variants were selected from array based data generated by the IAMDGC[3]. Fritsche *et al.*[3] showed these seven rare variants to be independently associated with AMD: *CFH* rs121913059 (p.Arg1210Cys), *CFI* rs141853578 (p.Gly119Arg), *C3* rs147859257 (p.Lys155Gln), and *C9* rs34882957 (p.Pro167Ser) and three non-coding variants in or near *CFH* (rs148553336, rs35292876, rs191281603).

The software package SAS, (Statistical Analysis System Institute, V9.2) was used to compare MAFs between the different geographical regions in a logistic regression analysis with Firth correction (S1 Supporting information)[14]. Furthermore, we estimated the mean allele frequency of each rare

genetic variant in each of the geographical regions including a 95% confidence interval (details provided in S1 Supporting information). To study a potential difference in effect size of each variant between the geographical regions, interaction analyses were performed using binary logistic regression models with SPSS statistics software (IBM SPSS Statistics, V22.0).

RESULTS

Demographic characteristics for each of the five geographical regions are shown in supporting information **S1 Table**. The characteristics of participants from the different regions were comparable, although the British study samples were slightly younger than the others, and the western European study samples included relatively more female participants compared to the remainder . These differences were comparable in both cases and controls.

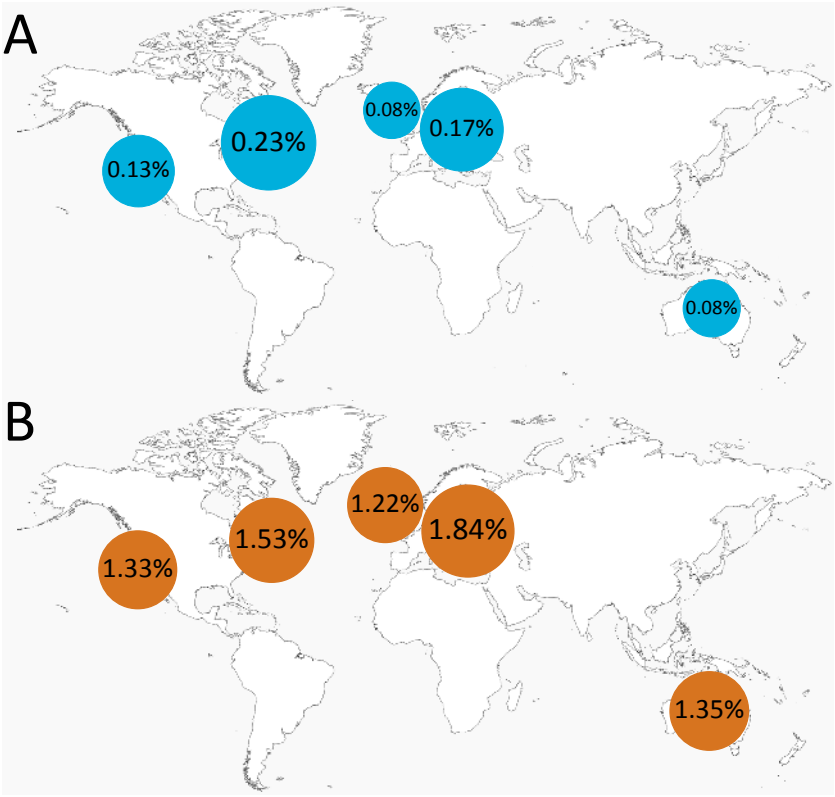

Figure 1. The two rare variants in *CFH* that are differently distributed variants among different geographical regions. Minor allele frequencies (in percentage) for *CFH* rs121913059 (A) and *CFH* rs35292876 (B). Variants mapped to geographical location (from left to right): western USA, eastern USA, Britain, western Europe, and Australia.

We analyzed the difference in distribution of the seven rare variants among case-control studies from eastern USA, western Europe, Britain, western USA and Australia using logistic regression analysis with Firth correction (**Table 2; Figure 1**), and observed a difference in distribution of variants *CFH* rs121913059 (p.Arg1210Cys, p=0.004) and *CFH* rs35292876 (p=0.001) across the different geographical regions. *CFH* rs121913059 was found at a higher frequency in eastern USA, especially compared to Britain and Australia (p=0.011 and p=0.003, respectively). *CFH* rs35292876 was found at a higher frequency in western Europe, compared to all other regions (ranging from p<0.001 in Britain to p=0.012 in Eastern USA). The other five variants were found to have similar allele frequencies among all geographical regions.

Table 2. Distribution and interaction analysis of seven rare AMD-associated genetic variants across five geographical regions.

	Difference in distribution between geographical regions#	**Interaction Analysis***	**Overall effect size‡**
	p-value	**p-value**	**OR (95%CI)**
CFH rs121913059 (p.Arg1210Cys)	**0.004**	0.665	24.2 (8.9-65.6)
CFI rs141853578 (p.Gly119Arg)	0.707	0.563	3.7 (2.5-5.7)
C3 rs147859257 (p.Lys155Gln)	0.665	0.680	2.8 (2.3-3.4)
C9 rs34882957 (p.Pro167Ser)	0.315	0.572	1.7 (1.5-2.0)
CFH rs148553336	0.053	0.015	0.5 (0.4-0.6)
CFH rs35292876	**0.001**	0.709	2.3 (2.0-2.6)
CFH rs191281603	0.735	0.980	0.9 (0.7-1.1)

#Logistic Regression with Firth correction. Individual Wald Chi-Square from likelihood ratio test for each of the variants across the geographical regions. *Interaction Analysis: Effect sizes in entire study and interaction analysis to study potential differences in effect size between cohorts. ‡Overall effect size adjusted for geographical region. Bold values: p-value considered significant after Bonferroni correction (p<0.007).

The difference in distribution is also reflected by the estimated MAFs of each variant in the different geographical regions (**S2 Table**). The allele frequency of *CFH* rs121913059 is nearly three times higher in eastern USA than in Britain and Australia. Noteworthy is the near absence of this risk variant in control individuals without AMD, indicating that the difference in distribution appears to be driven solely by AMD individuals (**S3 Table**).

To determine if the effect size was influenced by geographical region we performed interaction analyses for each variant. We observed that the risk associated with each specific rare variant is independent of geographical region (**Table 2**). Overall effect sizes of the rare variants are comparable to the effect sizes reported in the IAMDGC study[3].

DISCUSSION

The distribution of rare *CFH* variants rs121913059 (p.Arg1210Cys) and rs35292876 was significantly different between several of the studied geographical regions. This confirms differences reported in previous studies for the *CFH* rs121913059 variant[2-11,13,15] (**Table 1**). *CFH* rs121913059 was first associated with AMD in study from the USA[2], however the association was not consistently replicated in Dutch/German[7], Icelandic[8], Japanese[10] and Chinese[11] studies. In this study we confirmed the hypothesis that rare variants can be differently distributed among geographical regions but, as expected, the risk estimates are comparable across the geographical regions.

In AMD, a difference in geographical distribution has already been described for common risk haplotypes of *CFH* and *ARMS2* genes, which are the most prominent common genetic AMD risk factors[3]. While Asian populations report a lower frequency of *CFH* risk haplotypes, the opposite holds true for the *ARMS2/HTRA1* risk haplotype which is more prevalent in Asians compared to Caucasian populations[16,17]. These patient and population specific variations have implications for genetic counseling and carrier screening in both diagnostic and research settings.

Besides single variant associations, a significant burden of rare variants in the *CFH* and *CFI* genes has been reported for AMD[3,15]. The disease burden in these genes is attributed to the cumulative effect of rare coding variants, some of which are identified in multiple studies, while others are restricted to a single population or even a single patient[18]. Carriers of specific rare genetic variants in the complement genes that increase complement activation may benefit more from complement inhibiting therapy than those who do not carry such variants[18]. Personalized treatment aiming at complement activating rare variants in clinical trials may only be applicable to specific populations where these variants are sufficiently common.

It is likely that additional rare variants, other than *CFH* Arg1210Cys and rs35292876, fluctuate in frequency among geographical regions. To identify these variants, additional large sequencing studies will need to be performed in populations originating from diverse geographic regions. Up to now, large sequencing initiatives are predominantly of North American or European origin, and sample sizes for non-European-descent population are limited[3,19]. Recruiting case-control studies from other geographical regions and ancestries could allow for identification of

novel highly penetrant rare variants implicated in AMD pathogenesis. These variants may be located in known AMD pathways, such as the complement system, or novel pathways[20].

In conclusion, we demonstrated that rare AMD-associated variants *CFH* rs121913059 and rs35292876 are differently distributed among different geographical regions. These results emphasize the importance of identifying population-specific rare variants in AMD.

SUPPLEMENTARY INFORMATION

S1 Supporting information: Code for the software package SAS for a logistic regression analysis and Firth's bias correction.

```
*Load raw dataset and implement filter criteria;
*The following commands will analyze only variant CFH_Arg1210Cys;
libname 'name';
data geographic;
set lees.'name';
if include=1;
run;
proc print data=geographic;
run;

*Create variables to summarize data per geographical region;
data Add;
length samplename $27;
samplename = 'fake';
continent=1;
output;
samplename = 'fake';
continent=3;
output;
samplename = 'fake';
continent=4;
output;
samplename = 'fake';
continent=5;
output;
samplename = 'fake';
continent=6;
output;run;
proc print data=Add;
run;

data geographic2;
set geographic Add;
run;
```

```
*Logistic regression with Firth correction for each of the regions as reference;
*Using Proc logistic to suppresses the display of results;
*For more information check http://support.sas.com/en/support-home.html ;
proc logistic descending data =geographic2;
class continent (param=ref ref='1') CFH_Arg1210Cys ;
model CFH_Arg1210Cys = continent/cl firth;
output out=geographic_out predicted=pred lower=pred_low upper=pred_up;
run;
proc logistic descending data =geographic2;
class continent (param=ref ref='3') CFH_Arg1210Cys ;
model CFH_Arg1210Cys = continent/cl firth;
run;
proc logistic descending data =geographic2;
class continent (param=ref ref='4') CFH_Arg1210Cys ;
model CFH_Arg1210Cys = continent/cl firth;
run;
proc logistic descending data =geographic2;
class continent (param=ref ref='5') CFH_Arg1210Cys ;
model CFH_Arg1210Cys = continent/cl firth;
run;
proc logistic descending data =geographic2;
class continent (param=ref ref='6') CFH_Arg1210Cys ;
model CFH_Arg1210Cys = continent/cl firth;
run;

* Calculate estimated allele frequency per geographical region;
data geographic_out2;
set geographic_out;
if samplename='fake';
pred_low=pred_low*100;
pred_up=pred_up*100;
pred=pred*100;
keep continent pred pred_up pred_low;
run;
proc print data=geographic_out2;
var continent pred_low pred pred_up;
run;
```

S1 Table. Demographic characteristics of AMD cohorts grouped in five geographical regions.

		Eastern USA[1]	Western Europe[2]	Britain[3]	Western USA[4]	Australia[5]
Participants (n)		18454	6590	4329	4226	5983
Mean Age (years ± SD)		74.0 ± 9.2	74.1 ± 8.2	69.6 ± 10.4	74.8 ± 10.0	74.3 ± 9.7
Gender	Male (%)	7934 (43.0%)	2512 (38.1%)	1743 (40.3%)	1875 (44.4%)	2531 (42.3%)
	Female (%)	10520 (57.0%)	4078 (61.9%)	2586 (59.7%)	2351 (55.6%)	3452 (57.7%)
AMD status	AMD (%)	11564 (62.7%)	3865 (58.6%)	2125 (49.1%)	2061 (48.8%)	2449 (40.9%)
	No AMD (%)	6890 (37.3%)	2725 (41.4%)	2204 (50.9%)	2165 (51.2%)	3534 (59.1%)

[1)]Eastern USA: AREDS, BDES, CWRU, Marshfield, Vanderbilt, Miami, Michigan, Pittsburgh, Pennsylvania, Baltimore. [2)]Western Europe: Regensburg, Rotterdam, Creteil, Paris, Bonn, Cologne, UMCN. [3)]Britain: Cambridge, Southampton, NHS_HPF, Edinburgh. [4)]Western USA: University California, UCSD, Oregon. [5)] Australia: Westmead, UWA/LEI/ Flinders and Melbourne.

S2 Table. Overall estimated mean MAF of seven rare AMD-associated genetic variants across five geographical regions.

	Eastern USA	Western Europe	Britain	Western USA	Australia
CFH rs121913059	0.229 (0.185-0.283)	0.178 (0.119-0.267)	0.087 (0.042-0.177)	0.136 (0.076-0.242)	0.088 (0.048-0.161)
CFI rs141853578	0.197 (0.156-0.247)	0.163 (0.107-0.249)	0.249 (0.163-0.379)	0.184 (0.112-0.302)	0.213 (0.145-0.314)
C3 rs147859257	0.803 (0.717-0.900)	0.907 (0.758-1.084)	0.918 (0.738-1.143)	0.917 (0.734-1.144)	0.840 (0.691-1.020)
C9 rs34882957	1.196 (1.090-1.312)	1.286 (1.107-1.493)	1.276 (1.060-1.536)	1.366 (1.140-1.637)	1.065 (0.896-1.266)
CFH rs148553336	0.630 (0.554-0.716)	0.573 (0.457-0.717)	0.815 (0.645-1.027)	0.503 (0.373-0.679)	0.740 (0.601-0.910)
CFH rs35292876	1.535 (1.414-1.665)	1.840 (1.624-2.084)	1.230 (1.018-1.485)	1.331 (1.107-1.599)	1.350 (1.158-1.573)
CFH rs191281603	0.321 (0.268-0.384)	0.391 (0.297-0.513)	0.295 (0.200-0.434)	0.337 (0.234-0.486)	0.313 (0.228-0.431)

Calculated by SAS for each variant in percentage separated by geographical region including 95% confidence interval

S3 Table: Minor allele frequencies (%) of seven rare AMD-associated genetic variants across five geographical regions stratified by AMD status.

		Eastern USA	Western Europe	Britain	Western USA	Australia
	AMD	0.359	0.272	0.165	0.243	0.204
CFH rs121913059	No AMD	0.007	0.037	0.000	0.023	0.000
	AMD	0.259	0.259	0.353	0.291	0.388
CFI rs141853578	No AMD	0.087	0.018	0.136	0.069	0.085
	AMD	1.020	1.216	1.365	1.431	1.450
C3 rs147859257	No AMD	0.435	0.459	0.476	0.416	0.410
	AMD	1.397	1.630	1.647	1.698	1.307
C9 rs34882957	No AMD	0.856	0.789	0.907	1.039	0.891
	AMD	1.020	1.216	1.365	1.431	1.450
C3 rs147859257	No AMD	0.435	0.459	0.476	0.416	0.410
	AMD	0.519	0.349	0.376	0.291	0.286
CFH rs148553336	No AMD	0.813	0.881	1.225	0.693	1.047
	AMD	1.889	2.393	1.765	1.868	2.103
CFH rs35292876	No AMD	0.936	1.046	0.703	0.808	0.821
	AMD	0.307	0.336	0.259	0.315	0.286
CFH rs191281603	No AMD	0.341	0.459	0.318	0.346	0.325

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Eveline Kersten
Maartje J. Geerlings
Anneke I. den Hollander
Eiko K. de Jong
Sascha Fauser
Tunde Peto
Carel B. Hoyng

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6

PHENOTYPE CHARACTERISTICS OF PATIENTS WITH AGE-RELATED MACULAR DEGENERATION CARRYING A RARE VARIANT IN THE COMPLEMENT FACTOR H GENE

ABSTRACT

Importance: Rare variants in the complement factor H (*CFH*) gene and their association with age-related macular degeneration (AMD) have been described. However, there is limited literature on the phenotypes accompanying these rare variants. Phenotypical characteristics could help ophthalmologists select patients for additional genetic testing.

Objective: To describe the phenotypical characteristics of patients with AMD carrying a rare variant in the *CFH* gene.

Design, setting and participants: In this cross-sectional study, we searched the genetic database of the department of ophthalmology at the Radboudumc (tertiary ophthalmologic referral center) and the European Genetic Database for patients with AMD with a rare genetic variant in the *CFH* gene. Patient recruitment took place from March 30, 2006, to February 18, 2013, and data were analyzed from November 30, 2015, to May 8, 2017. Phenotypical features on fundus photographs of both eyes of patients were graded by two independent reading center graders masked for carrier status.

Main Outcomes and Measures: Differences in phenotypical characteristics between rare variant carriers and noncarriers were analyzed using univariable generalized estimated equations logistic regression models accounting for intereye correlation.

Results: Analyses included 100 eyes of 51 patients with AMD carrying a *CFH* variant (mean [SD] age, 66.7 [12.1] years; 64.7% female) and 204 eyes of 102 age-matched noncarriers (mean [SD] age, 67.1 [11.8] years; 54.9% female). Carrying a rare pathogenic *CFH* variant was associated with larger drusen area (odds ratio range, 6.98 [95% CI, 2.04-23.89] to 18.50 [95% CI, 2.19-155.99]; P = 0.002), presence of drusen with crystalline appearance (odds ratio, 3.24; 95% CI, 1.24-8.50; P = 0.02), and drusen nasal to the optic disc (odds ratio range, 4.03 [95% CI, 1.70-9.56] to 7.42 [95% CI, 0.65-84.84]; P = 0.003).

Conclusions and Relevance: Identification of rare *CFH* variant carriers may be important for upcoming complement-inhibiting therapies. Patients with an extensive drusen area, drusen with crystalline appearance, and drusen nasal to the optic disc are more likely to have a rare variant in the *CFH* gene. However, it is not likely that carriers can be discriminated from noncarriers based solely on phenotypical characteristics from color fundus images. Therefore, ophthalmologists should consider genetic testing in patients with these phenotypic characteristics in combination with other patient characteristics, such as early onset, cuticular drusen on fluorescein angiography, and family history of AMD.

INTRODUCTION

Age-related macular degeneration (AMD) is a common multifactorial eye disease in Western countries,[1] however the exact pathophysiology of the disease is not yet completely understood. Environmental factors, such as age and smoking,[2,3] and both common and rare genetic variants have been identified as risk factors for AMD.[4] A large number of these genetic variants are located in genes encoding components of the complement system. Additionally, higher local and systemic complement activity has been reported in patients with AMD compared with control individuals.[5-7] Together, these findings implicate a pivotal role of the complement system in AMD.

Rare genetic variants located in the complement factor H (*CFH*) gene are among the variants that confer the highest risk for AMD.[4,8,9] The *CFH* gene encodes factor H (FH), a regulator of the alternative pathway of the complement system. Factor H inhibits the C3-convertase (C3bBb) and also acts as cofactor for factor I-mediated inactivation of C3b,[10] leading to decreased activity and thereby preventing overactivation of the complement system. Several studies showed lower systemic FH levels in patients carrying a rare *CFH* variant.[10,11] Furthermore, functional studies have reported an altered function of FH in patients carrying a rare variant in *CFH* resulting in increased complement activation despite normal systemic FH levels.[9,12,13] While antivascular endothelial growth factor treatment is available for neovascular AMD, there is currently no effective treatment available for the early and atrophic stages of AMD. Because the complement system plays an important role in AMD pathogenesis, therapies targeting different components of the complement system are being developed. Currently, a number of phase 2/3 clinical trials are in progress, and so far two phase 2 trials have been completed with mixed results.[9,14,15] The Complement Inhibition With Eculizumab for the Treatment of Non-Exudative Age-Related Macular Degeneration (COMPLETE) study did not show decreased atrophy progression after administration of eculizumab,[16] while the MAHALO study showed beneficial effect of lampalizumab treatment on reducing atrophy progression.[17]

With upcoming therapies targeting the complement system, it may be important to identify the patients who will most likely benefit from these therapies. Patients carrying a rare variant in the *CFH* gene seem to be a very suitable patient group for complement inhibiting therapies because of the associated functional consequences on complement activation.[12] However, it is expensive to genetically screen every patient with AMD in a diagnostic setting; therefore, it is desirable to preselect cases for genotyping based on phenotype. Unfortunately, there is limited literature on the phenotypes accompanying these *CFH* variants. Previously, a higher burden of extramacular drusen was reported in families carrying rare *CFH* variants compared with unrelated AMD cases; however, other distinct phenotypical characteristics were not described.[11] Another study described phenotypical characteristics in a more detailed manner,

but only included individuals carrying the rare p.Arg1210Cys variant in *CFH*.[18] We hypothesize that all pathogenic *CFH* variants share phenotypical characteristics owing to their functional influences on FH. Detailed characterization of phenotypes caused by a broad spectrum of rare *CFH* variants is lacking. Therefore, we aim to describe the phenotypical characteristics of patients with AMD carrying a rare variant in the *CFH* gene. A distinct phenotype description of these *CFH* carriers will enable ophthalmologists to select patients for additional genetic testing and complement-inhibiting therapies more efficiently.

METHODS

Study Population

In this retrospective cross-sectional study, we searched the genetic database of the department of ophthalmology at the Radboud university medical center, Nijmegen, the Netherlands (Radboudumc) and the European Genetic Database (EUGENDA), a multicenter database for clinical and molecular analysis of AMD, for individuals with a rare genetic variant in the *CFH* gene. Patient recruitment took place from March 30, 2006, to February 18, 2013. We selected AMD cases carrying protein-altering variants with a population frequency of less than 1%. We defined AMD as the presence of at least 10 small drusen (<63 μm) and pigmentary changes, intermediate or large drusen (≥63 μm), or late AMD, including subfoveal geographic atrophy (GA) and/or choroidal neovascularization (CNV) in at least one eye on color fundus images. Details of this classification are described elsewhere.[19]

In total, 51 patients, with 33 different *CFH* variants, were identified and included in this study, hereafter referred to as carriers. Additionally, for each carrier, we selected from the European Genetic Database two similarly aged AMD cases (±2 years) without a rare genetic variant associated with AMD; these cases were defined as noncarriers (n = 102). For two carriers color fundus images of only one eye were available; therefore, final analyses included 100 eyes of 51 carriers and 204 eyes of 102 noncarriers. All participants indicated to be of European descent. Written informed consent was provided by all participants. The study was approved by the local ethics committee on research involving human participants, Commissie Mensgebonden Onderzoek Regio Arnhem-Nijmegen, and the local committee of University Hospital Cologne and was performed in accordance with the tenets of the Declaration of Helsinki.

Genotyping

Whole-exome sequencing (WES) and/or Sanger sequencing was previously performed. For both approaches, DNA was extracted from venous blood using standard procedures. Most *CFH* carriers (n = 42) were identified through WES. Preparation and sequencing of the DNA samples were done as previously described.[12] In short, exome capture Nimblegen SeqCap EZ V2 kit

(Roche) paired-end sequencing was performed on an Illumina HiSeq2000 sequencer using TruSeq V3 chemistry (Illumina) followed by downstream quality control and genotyping of the samples. For this study, WES data were filtered specifically for the *CFH* gene (HUGO Gene Nomenclature Committee ID: 4883; NM_000186). Additional filtering steps on the data were implemented to select genetic variants that result in a splice-site or protein change (non-synonymous) as these variants are more likely to be pathogenic. We focused on rare genetic variants only (minor allele frequency ≤ 1%) as based on the Exome Aggregation Consortium (ExAC) database, specifically the non-Finnish European population.[20] Individual variants were confirmed with Sanger sequencing using primers designed by Primer3 software.[21] The remainder of *CFH* carriers (n = 9) was identified through conventional Sanger sequencing of the entire *CFH* gene as described in detail previously.[22] We excluded rare *CFH* variants with a described protective effect in case-control analyses (c.2850G>T p.Gln950His) or a likely benign effect in functional studies (c.2669G>T p.Ser890Ile, c.2867C>T p.Thr956Met, c.3019G>T p.Val1007Leu).[9] All *CFH* variants included in this study are described in the eTable in the in the Supplement

For all noncarriers, WES data were available and screened for rare genetic variants in the *CFH, CFI, C3* and *C9* genes associated with AMD. Only individuals without any rare variant in the *CFH* gene or a described pathogenic rare variant in the other AMD-associated genes[9] were included in this study as noncarriers.

Image Assessment

Digital 35° or 40° field of view color fundus photographs centered on the fovea were performed with a Topcon TRC 50IX camera (Topcon Corporation) or Canon 60 UVi fundus camera (Canon), respectively. Color fundus photographs were analyzed for this study by two senior graders from an independent reading center (Moorfields Eye Hospital, London, England, UK) according to a standardized grading protocol. The following fundus features were assessed: predominant type of drusen, largest type of drusen in the central field, percentage of the area of the Early Treatment Diabetic Retinopathy Study (ETDRS) grid covered with drusen, presence of extramacular drusen (defined as drusen outside the ETDRS grid), drusen nasal to the optic disc, reticular drusen, drusen with crystalline appearance, serogranular/serous drusen pigment epithelium detachment, pigmentary abnormalities, geographic atrophy, or signs of neovascularization.

Statistical analysis

Data were analyzed from November 30, 2015 to May 8, 2017. Demographic characteristics of the two study groups were compared using one-way analysis of variance or the χ^2 test. Phenotypical characteristics were individually assessed using binary logistic regression models. Generalized estimating equations procedures were used to correct for the fellow eye. To compare the

frequencies of late AMD subtypes between carriers and noncarriers, we performed a χ^2 test based on the more severely affected eye of each patient. In case both geographic atrophy and choroidal neovascularization were present in an individual, it was classified as mixed late AMD. A phenotypic risk score for each eye was calculated as the sum of regression coefficients of all individual phenotypical characteristics resulting from univariable generalized estimating equations logistic regression analyses. A receiver operating characteristic curve was obtained and the area under the curve was measured for this risk score. Finally, symmetry between eyes was calculated as follows: number of equal phenotypical characteristics between right and left eye divided by the number of phenotypical characteristics graded times 100%. All statistical analyses were performed using SPSS statistics software (released 2013; IBM SPSS Statistics for Windows, Version 22.0; IBM Corp).

RESULTS

In total, 100 eyes of 51 carriers and 204 eyes of 102 noncarriers were included for analyses. Demographic and environmental characteristics were comparable between carriers and noncarriers (**Table 1**). The frequency of common genetic variants in *CFH, ARMS2,* and *C3* seems to be slightly higher in noncarriers compared with carriers. However, the minor allele frequencies of these common variants in noncarriers are comparable with the general AMD population.[23] This may imply that carriers of rare *CFH* variants are less burdened by common AMD risk variants and that their AMD risk is rather attributable to the rare variants.

When comparing the fundus features by carrier status, the odds of carrying any rare *CFH* variant increases with increasing drusen area within the ETDRS grid (odds ratio [OR] up to 6.85 when more than 50% of the ETDRS grid is covered with drusen, P = 0.004), and with the presence of serogranular/serous drusen pigment epithelium detachment (OR, 4.74; 95% CI, 1.30-17.31; P = 0.02). Additionally, drusen deposition in rare variant carriers is often not limited to the central retina; these carriers tend to have extramacular drusen (80.8%) and drusen nasal to the optic disc (43.8%) more frequently than noncarriers (73.4% and 35.1%, respectively), although these differences were not significant. The association of all assessed fundus features of carriers and noncarriers are shown in **Table 2**.

Table 1: General Characteristics of the Study Groups.

Characteristic	No. (%)	
	Noncarriers (n = 102; 204 eyes)	**Carriers (n = 51; 100 eyes)**
Age at participation, mean (SD), y	67.1 (11.8)	66.7 (12.1)
Sex		
- Male	46 (45.1)	18 (35.3)
- Female	56 (54.9)	33 (64.7)
Smoking status		
- Never	21 (22.3)	16 (39.0)
- Past	53 (56.4)	17 (41.5)
- Current	20 (21.3)	8 (19.5)
BMI, mean (SD)	26.1 (4.1)	26.5 (4.2)
Family history for AMD		
- Yes	52 (58.4)	27 (67.5)
- No	37 (41.6)	13 (32.5)
Common genetic variants, No. of minor allels/total No. of alleles (MAF %)		
- *ARMS2*, rs10490923, T	75/176 (42.6)	17/74 (23.0)
- *CFH*, rs1061170, C	99/176 (56.3)	29/76 (38.2)
- *C3*, rs2230199, G	49/174 (28.2)	10/76 (13.2)

Abbreviations: ARMS2, age-related maculopathy susceptibility 2; BMI, body mass index (calculated as weight in kilograms divided by height in meters squared); C3, complement component 3; CFH, complement factor H; MAF, minor allele frequency.

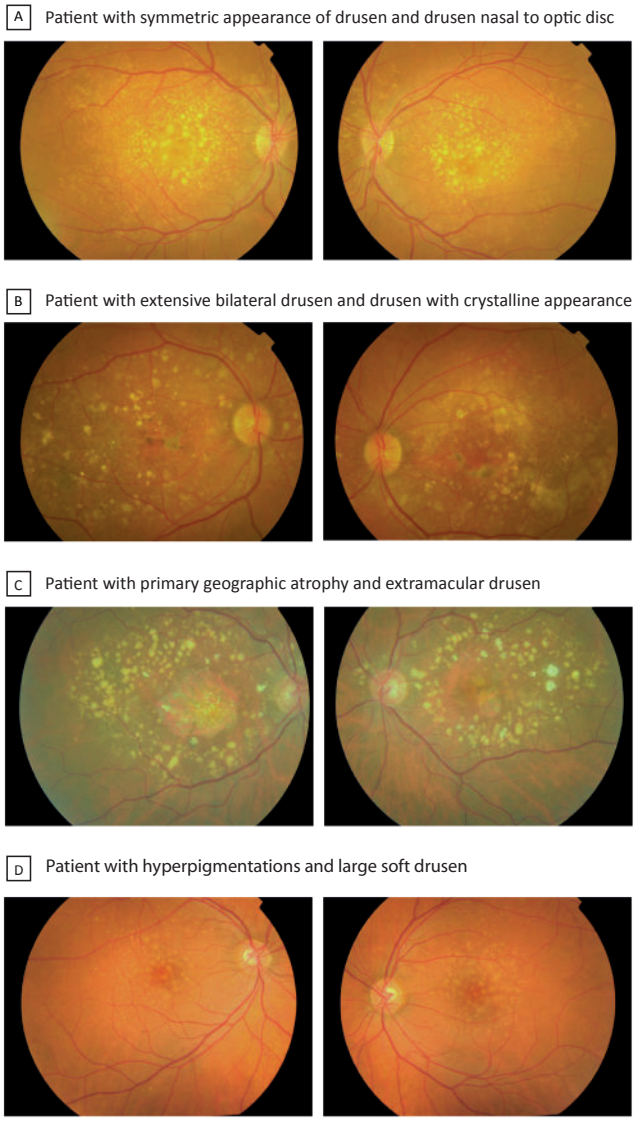
Because the carrier group contains both rare variants known to be associated with AMD and rare variants of unknown clinical significance, we repeated the analyses with stricter inclusion criteria comparing only cases carrying a known pathogenic variant (n = 25) with noncarriers (**Table 3**). Known pathogenic variants included rare *CFH* variants associated with AMD in case-control or segregation analyses or with a described functional effect. This subanalysis showed an even higher association between drusen area within the ETDRS grid and rare pathogenic *CFH* variant carriers (OR range, 6.98 [95% CI, 2.04-23.89] to 18.50 [95% CI, 2.19-155.99]; P = 0.002). Additionally, intermediate and large drusen located nasal to the optic disc (OR range, 4.03 [95% CI, 1.70-9.56] to 7.42 [95% CI, 0.65-84.84]; P = 0.003) and the presence drusen with crystalline appearance (OR, 3.24; 95% CI, 1.24-8.50; P = 0.02) were significantly associated with carrying a rare pathogenic *CFH* variant. Subanalysis of late AMD cases only (n = 71) showed a higher frequency of late atrophic AMD in rare pathogenic variant carriers (57.1%) compared with noncarriers (28.1%), although this was not significantly different (P = 0.12). Notably, the association between serogranular/serous drusen pigment epithelium detachment and carrier status did not remain significant but still tended to increase the odds of carrying a rare *CFH* variant. Examples of color fundus photographs of carriers of rare *CFH* variants with the associated fundus features are displayed in **Figure 1**.

Table 2: Phenotypical Characteristics of Carriers and Noncarriers of Rare *CFH* Variants.

	No. of Eyes (%)			
Phenotypic characteristic	**Noncarrier (n = 204)**	**Carrier (n = 100)**	**Odds ratio (95% CI)[a]**	***P-value***
Predominant drusen type within ETDRS grid				
None or small drusen (<63 µm)	32 (15.7)	12 (12.0)	Reference	0.31
Intermediate drusen (63-125 µm)	107 (52.5)	45 (45.0)	1.12 (0.47-2.68)	
Large drusen (>125 µm)	65 (31.9)	43 (43.0)	1.76 (0.71-4.38)	
Largest drusen type within central field				
None or small drusen (<63 µm)	106 (52.5)	44 (44.9)	Reference	0.58
Intermediate drusen (63-125 µm)	77 (38.1)	44 (44.9)	1.38 (0.75-2.52)	
Large drusen (>125 µm)	19 (9.4)	10 (10.2)	1.27 (0.46-3.48)	
Proportion of grid area covered by drusen, %				
0-10	111 (54.4)	27 (27.3)	Reference	0.004[b]
10-25	61 (29.9)	41 (41.4)	2.76 (1.36-5.63)	
25-50	29 (14.2)	26 (26.3)	3.69 (1.58-8.58)	
>50	3 (1.5)	5 (5.1)	6.85 (1.37-34.37)	
Extramacular drusen				
Absent	54 (26.6)	19 (19.2)	Reference	0.27
Present	149 (73.4)	80 (80.8)	1.53 (0.72-3.24)	
Drusen nasal to the optic disc				
None or small drusen (<63 µm)	89 (65.0)	45 (56.3)	Reference	0.47
Intermediate drusen (63-125 µm)	46 (33.6)	32 (40.0)	1.38 (0.69-2.74)	
Large drusen (>125 µm)	2 (1.5)	3 (3.8)	2.97 (0.30-29.51)	
Reticular drusen				
Absent	163 (86.7)	83 (93.3)	Reference	0.14
Present	25 (13.3)	6 (6.7)	0.47 (0.18-1.27)	
Drusen with crystalline appearance				
Absent	178 (89.4)	76 (81.7)	Reference	0.15
Present	21 (10.6)	17 (18.3)	1.90 (0.80-4.48)	
SPED				
Absent	199 (97.5)	84 (89.4)	Reference	0.02
Present	5 (2.5)	10 (10.6)	4.74 (1.30-17.31)	
RPE pigmentation				
Absent	84 (45.4)	31 (32.3)	Reference	0.08
Present	101 (54.6)	65 (67.7)	1.74 (0.93-3.27)	
Geographic atrophy				

Absent	154 (76.2)	65 (70.7)	Reference	0.42
Present	48 (23.8)	27 (29.3)	1.33 (0.66-2.69)	
Neovascular AMD				
Absent	144 (72.4)	79 (82.3)	Reference	0.12
Present	55 (27.6)	17 (17.7)	0.56 (0.28-1.15)	

Abbreviations: AMD, age-related macular degeneration; *CFH*, complement factor H; ETDRS, Early Treatment Diabetic Retinopathy Study; RPE, retinal pigment epithelium; SPED, serogranular/serous drusen pigment epithelium detachment. [a] The presented odds ratios result from univariable generalized estimating equations logistic regression analyses. [b] *P*-value remained significant after Bonferroni correction for multiple testing.



A) A woman in her 50s (CFHc.2537A>G, p.Gln846Arg) with a symmetric appearance of extensive drusen deposition within the Early Treatment Diabetic Retinopathy Study grid extending beyond the inferior and superior retinal arcades and nasal to the optic disc in both eyes.

B) A man in his 60s (CFHc.550delA, p.Ile184Leufs*33) with extensive bilateral drusen deposition inside and outside the Early Treatment Diabetic Retinopathy Study grid and nasal to the optic disc, presence of drusen with crystalline appearance, and hypopigmentations and hyperpigmentations.

C) Woman in her 70s (CFH c.524G>A, p.Arg175Gln) with primary geographic atrophy surrounded by predominantly large drusen, some with crystalline appearance, beyond the retinal arcades and the optic disc.

D) A man in his 40s (CFH c.1198C>A, p.Gln400Lys) with hyperpigmentations and mainly centrally located large soft drusen but also extending to the peripheral retina.

Figure 1. Color Fundus Photographs of Carriers of Rare Variants in the Complement Factor H (*CFH*) Gene.

Table 3. Associations of phenotypical characteristics with confirmed pathogenic rare *CFH* variants.

Phenotypic characteristic	No. of Eyes (%)		Odds ratio (95% CI)[a]	*P-value*
	Noncarrier (n = 204)	Carrier (n = 48)		
Predominant drusen type within ETDRS grid				
None or small drusen (<63 µm)	32 (15.7)	4 (8.3)	Reference	0.06
Intermediate drusen (63-125 µm)	107 (52.5)	18 (37.5)	1.35 (0.34-5.38)	
Large drusen (>125 µm)	65 (31.9)	26 (54.2)	3.20 (0.80-12.75)	
Largest drusen type within central field				
None or small drusen (<63 µm)	106 (52.5)	24 (50.0)	Reference	0.97
Intermediate drusen (63-125 µm)	77 (38.1)	19 (39.6)	1.16 (0.29-4.66)	
Large drusen (>125 µm)	19 (9.4)	5 (10.4)	1.09 (0.50-2.39)	
Proportion of grid area covered by drusen, %				
0-10	111 (54.4)	6 (12.5)	Reference	0.002[b]
10-25	61 (29.9)	23 (47.9)	6.98 (2.04-23.89)	
25-50	29 (14.2)	16 (33.3)	10.21 (2.85-36.59)	
>50	3 (1.5)	3 (6.3)	18.50 (2.19-155.99)	
Extramacular drusen				
Absent	54 (26.6)	6 (12.5)	Reference	0.11
Present	149 (73.4)	42 (87.5)	2.54 (0.80-8.04)	
Drusen nasal to the optic disc				
None or small drusen (<63 µm)	89 (65.0)	12 (30.8)	Reference	0.003[b]
Intermediate drusen (63-125 µm)	46 (33.6)	25 (64.1)	4.03 (1.70-9.56)	
Large drusen (>125 µm)	2 (1.5)	2 (5.1)	7.42 (0.65-84.84)	
Reticular drusen				
Absent	163 (86.7)	43 (93.5)	Reference	0.23
Present	25 (13.3)	3 (6.5)	0.46 (0.13-1.64)	
Drusen with crystalline appearance				
Absent	178 (89.4)	34 (72.3)	Reference	0.02
Present	21 (10.6)	13 (27.7)	3.24 (1.24-8.50)	
SPED				
Absent	199 (97.5)	43 (91.5)	Reference	0.11
Present	5 (2.5)	4 (8.5)	3.70 (0.74-18.63)	
RPE pigmentation				
Absent	84 (45.4)	13 (28.3)	Reference	0.10
Present	101 (54.6)	33 (71.7)	2.11 (0.87-5.14)	

Geographic atrophy				
Absent	154 (76.2)	28 (65.1)	Reference	0.23
Present	48 (23.8)	15 (34.9)	1.72 (0.71-4.14)	
Neovascular AMD				
Absent	144 (72.4)	38 (82.6)	Reference	0.23
Present	55 (27.6)	8 (17.4)	0.55 (0.21-1.47)	

Abbreviations: AMD, age-related macular degeneration; CFH, complement factor H; ETDRS, Early Treatment Diabetic Retinopathy Study; RPE, retinal pigment epithelium; SPED, serogranular/serous drusen pigment epithelium detachment. a The presented odds ratios result from univariable generalized estimating equations logistic regression analyses. b P-value remained significant after Bonferroni correction for multiple testing.

Overall, rare *CFH* variant carriers tend to have more and larger drusen, and drusen are more often located outside the ETDRS grid. However, not all of these analyzed phenotypical characteristics individually reach statistical significance. Based on the findings in **Table 3**, we calculated a phenotypic risk score for each eye including all assessed phenotypical characteristics (**eFigure 1 in the Supplement**). The mean (SD) phenotypic risk score in carriers (4.35 [2.0]) was significantly higher compared with noncarriers (2.32 [2.5]), although the ability to accurately discriminate between eyes of carriers of pathogenic *CFH* variants and noncarriers based on the phenotypic risk score was limited (area under the curve, 0.75; 95% CI, 0.65-0.85; **eFigure 2 in the Supplement**). Similar results were obtained when including only the highest phenotypic risk score for each patient (area under the curve, 0.75; 95% CI, 0.61-0.88).

Finally, for every patient, the grade of symmetry between eyes was determined based on the number of equal characteristics. Each study group showed a high grade of symmetry between the eyes (79.9% in noncarriers vs 79.1% in carriers of pathogenic variants) and the groups were not significantly different (P = 0.85).

DISCUSSION

In this study, we aimed to describe the phenotypical characteristics of patients with AMD carrying a rare variant in the *CFH* gene. Overall, rare *CFH* variant carriers have a more severe phenotype with more and larger drusen, often extending to the peripheral retina. Larger drusen area within the ETDRS grid and drusen located nasal to the optic disc were significantly associated with patients with AMD carrying a rare pathogenic *CFH* variant. These findings are in line with previous studies reporting extensive macular drusen accumulation and presence of extramacular drusen in patients with AMD carrying the *CFH* p.Arg1210Cys variant[18] and other rare *CFH* variants.[11]

In addition, we report an association between the presence of drusen with crystalline appearance and carrying a rare variant in *CFH*. Drusen with crystalline appearance, also known as refractile or calcified drusen, have a characteristic glistening appearance on color fundus imaging and have been associated with the development of geographic atrophy.[24,25] Thus, these patients might be at higher risk for developing geographic atrophy compared with noncarriers. In the current study, rare *CFH* variant carriers seem to develop geographic atrophy more often than choroidal neovascularization, as was already observed in rare variant carriers of other complement genes (*CFI, C3*, and *C9*).[26] However, probably owing to the small number of patients with late AMD, this difference was not significant.

From literature, it is known that *CFH* carriers usually have an earlier age at onset.[8,11,13,22,26,27] Owing to our study design a lower age at onset in rare variant carriers could not be analyzed. Our study was merely designed to analyze phenotypical differences between rare *CFH* carriers and noncarriers; therefore, age-matched noncarriers were selected. As a consequence, no difference in age at onset could be observed. However, assessing the age at onset remains an important clue for ophthalmologists when considering (rare) genetic variants in a patient.
Familial burden is also known to be associated with rare *CFH* variant carriers.[11,13,18,26,28] Although the number of carriers with a family history of AMD (64.7%) was not significantly different from noncarriers (53.9%), it must be emphasized that family history was obtained through interviewer-assisted questionnaires. From previous studies, it is known that *CFH* carriers often have asymptomatic family members[29,30] and, therefore, it is plausible that the percentage of carriers with a family history of AMD is underestimated.

Assuming that rare protein-altering variants located in the *CFH* gene lead to similar phenotype, this study was not restricted to one or more specific *CFH* variants but included a wide variety of rare protein-altering *CFH* variants identified by WES or Sanger sequencing in our cohort. Therefore, our analyses also included some variants that were not described before in the literature. However, when limiting the analyses to confirmed pathogenic variants only, the associations between rare variant carriers and phenotypical characteristics become more pronounced. More information on pathogenicity of variants is therefore desirable. As prediction tools do not always correctly predict a genetic variant to be functionally impaired,[31,32] other large sequencing or functional studies are needed to confirm the clinical significance of these variants.

Limitations

Because of an overlap in phenotypical characteristics between carriers and noncarriers, even when including only confirmed pathogenic variants, the sample size of our study might be insufficient to detect small to moderate associations or associations with relatively infrequent features, such as serogranular/serous drusen pigment epithelium detachment and the

presence of geographic atrophy. Additionally, when correcting for multiple comparisons, only drusen area remained significantly associated with rare variant carriers, which is most likely the result of our small sample size.

Our study was also restricted by its retrospective design, therefore, for the analyses, we were limited to the images that were captured in the past. Peripheral fundus images and other image modalities were often lacking and therefore not taken into account in the current study. To assess to what extent drusen are located outside the central retina, imaging should preferably be extended to the peripheral retina. Additionally, certain phenotypical characteristics are better visualized with other imaging techniques (eg, cuticular drusen). Previously, *CFH* variants were identified in patients with the cuticular drusen subtype of AMD, and fluorescein angiography is considered the best modality to diagnose these type of drusen.[22,29,30] Furthermore, optical coherence tomography enables detailed visualization of the different retinal layer structures that are not visible on color fundus images, and has the advantage of three-dimensional image assessment. Future prospective studies could therefore benefit from assessing multiple image modalities and imaging of the peripheral retina.

CONCLUSION

Because patients with AMD carrying a rare *CFH* variant seem a very suitable group for upcoming complement-inhibiting therapies, identification of this subpopulation may be very important to direct choice of treatment. Our results indicate that patients with an extensive drusen area, drusen with crystalline appearance, and drusen nasal to the optic disc are more likely to have a rare genetic variant in the *CFH* gene. These phenotypical characteristics could aid ophthalmologists to select patients for genetic screening. However, it is unlikely that carriers can be discriminated from noncarriers based solely on phenotypical characteristics. Therefore, ophthalmologists should consider genetic testing in patients with extensive drusen deposition, drusen with crystalline appearance and/or drusen nasal to the optic disc in combination with other patient characteristics, such as an early age at onset, cuticular drusen on fluorescein angiography, and a positive family history for AMD.

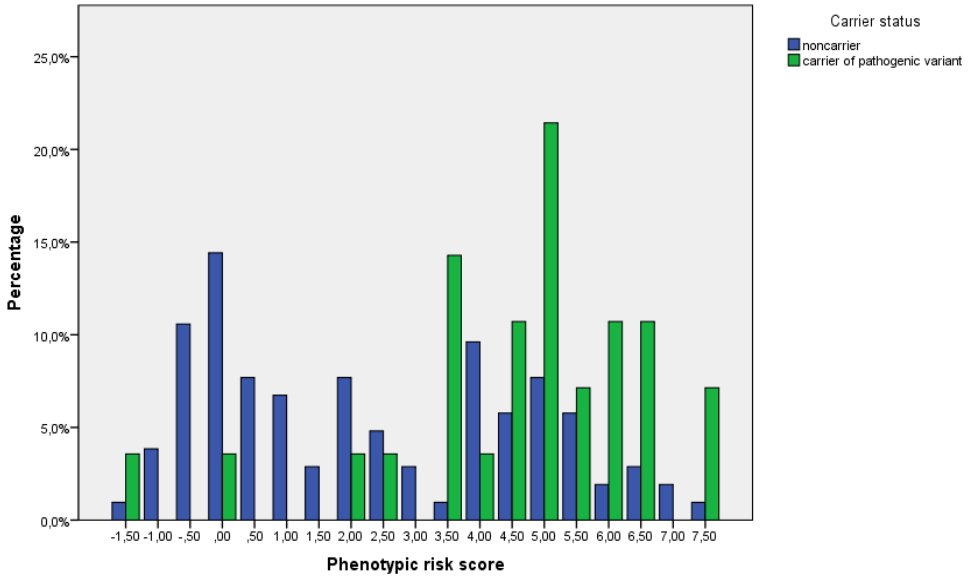
SUPPLEMENTARY CONTENT

eTable. Rare Genetic Variants Identified in the *CFH* Gene.

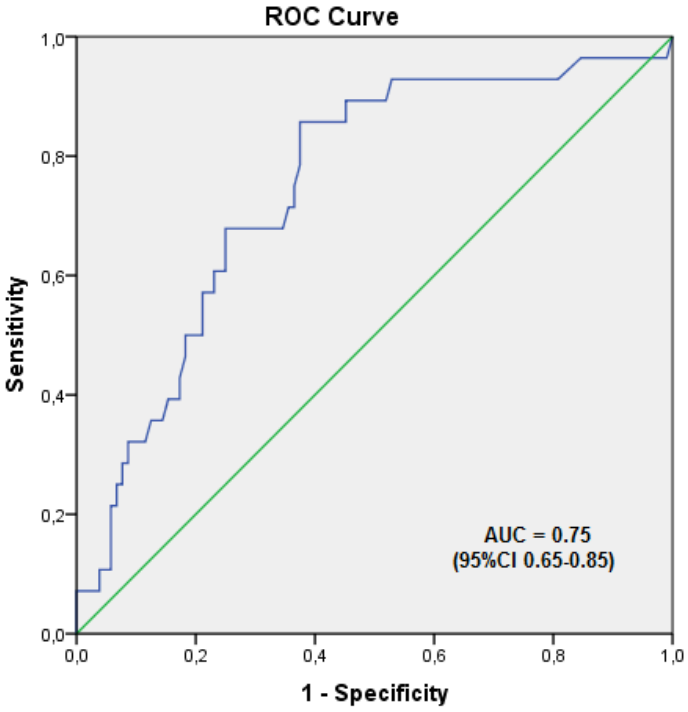
						Prediction algorithms			
No. of cases	**Genomic startposition**	**Nucleotide change**	**Protein change**	**SNP ID**	**ExAC[a]**	**SIFTb**	**Polyphen2 HDIVc**	**CADD PHREDd**	**Clinical significance**
Previously reported rare CFH variants									
1	196621254	c.7C>G	p.Leu3Val	rs139254423	0.03	T (0.26)	D (0.965)	6.682	Unknown[1,2]
1	196642221	c.172T>G	p.Ser58Ala	rs141336681	0.02	D (0.05)	B (0.151)	9.864	Risk (case-control analysis)[1,3,4]
1	196643098	c.350+6T>G	splice-donorsite	rs387906550	NA	NA	NA	NA	Risk (segregation analysis)[5]
1	196646659	c.481G>T	p.Ala161Ser	rs777300338	0.003	T (0.94)	P (0.923)	10.30	Unknown[2,3]
1	196646674	c.496C>T	p.Arg166Trp	.	NA	D (0.01)	D (1.0)	11.81	Unknown[3]
1	196646696	c.518C>G	p.Ala173Gly	.	NA	T (0.13)	B (0.209)	6.230	Unknown[2,6]
7	196646702	c.524G>A	p.Arg175Gln	.	NA	T (0.65)	B (0.005)	0.014	Risk (functional analysis)[2,7]
1	196646728	c.550delA	p.Ile184Leufs*33	.	NA	NA	NA	NA	Risk (segregation analysis)[8]
7	196646756	c.578C>T	p.Ser193Leu	.	NA	T (0.67)	D (1.0)	15.11	Risk (functional analysis)[2,7]
1	196648780	c.647T>C	p.Ile216Thr	rs183474263	NA	T (0.60)	B (0.005)	5.591	Unknown[2]
7	196654311	c.908G>A	p.Arg303Gln	rs766408580	NA	T (0.61)	D (0.976)	11.03	Unknown[1]
2	196659231	c.1198C>A	p.Gln400Lys	rs201671665	0.01	T (0.91)	B (0.04)	0.012	Risk (functional analysis)[1-4]
2	196659255	c.1222C>T	p.Gln408*	rs121913061	NA	T (0.22)	NA	15.49	Risk (segregation analysis)[5]
1	196683035	c.1507C>G	p.Pro503Ala	rs570523689	NA	T (0.23)	D (0.965)	12.43	Risk (case-control analysis)[3,4,9]
1	196684855	c.1652T>C	p.Ile551Thr	rs35453854	0.009	T (0.3)	D (0.999)	13.01	Unknown[4]
1	196694234_ 196694243	c.1697-17_-8	Splice-acceptor site	.	NA	NA	NA	NA	Unknown[8]
1	196695675	c.1949G>T	p.Gly650Val	rs143237092	0.03	T (0.28)	B (0.095)	13.53	Unknown[1,3,4,10]
1	196706001	c.2461C>T	p.His821Tyr	rs367687415	0.0002	T (1)	B (0.044)	0.106	Unknown[1,4]
1	196711077	c.3029C>T	p.Ala1010Val	.	NA	T (0.52)	B (0.002)	9.318	Unknown[3]

1	196712682	c.3234G>T	p.Arg1078Ser	rs121913062	0.007	T (0.76)	B (0.035)	15.09	Risk (segregation analysis)[5]
2	196716375	c.3628C>T	p.Arg1210Cys	rs121913059	0.03	D (0.03)	B (0.024)	15.48	Risk (case-control analysis)[1-4,10-15]
Novel rare CFH variants									
1	196642194	c.145A>G	p.Ile49Val	rs747546121	0.0015	T (0.65)	B (0.002)	0.230	Unknown
1	196642260	c.211T>A	p.Trp71Arg	.	NA	D (0.02)	D (1.0)	16.92	Unknown
1	196646750	c.572A>G	p.His191Arg	.	NA	T (0.35)	D (0.999)	12.52	Unknown
1	196648897	c.764G>A	p.Gly255Glu	rs771112278	0.0001	T (0.07)	D (1)	15.59	Unknown
3	196654303	c.900TG>T	p.Ala300Glnfs*22	.	NA	NA	NA	NA	Unknown
1	196659226	c.1193A>G	p.Tyr398Cys	rs765210362	NA	T (0.09)	D (0.999)	10.83	Unknown
1	196659281	c.1248C>G	p.Cys416Trp	.	NA	D (0)	D (1.0)	12.77	Unknown
1	196695729	c.2003C>T	p.Pro668Leu	rs764187411	0.003	T (0.31)	P (0.893)	13.81	Unknown
1	196697568	c.2329A>G	p.Ile777Val	rs761904009	0.0015	T (1)	B (0.001)	4.132	Unknown
1	196706077	c.2537A>G	p.Gln846Arg	.	NA	T (0.61)	B (0.004)	3.988	Unknown
3	196706112	c.2572T>A	p.Trp858Arg	.	NA	D (0)	D (1.0)	14.20	Unknown
1	196712608	c.3160G>A	p.Val1054Ile	rs757426928	0.0015	T (0.96)	P (0.955)	12.83	Unknown

[a] ExAC: Exome Aggregation Consortium. Frequencies presented here are based on a non-Finnish European population which is best comparable to our cohort. [b]SIFT: Sorting Intolerant from Tolerant. D: Deleterious (sift score ≤0.05); T: tolerated (sift score >0.05) [c]PolyPhen2 HDIV: Polymorphism Phenotyping version 2. D: Probably damaging (score ≥0.957), P: possibly damaging (0.453≤ score ≤0.956); B: benign (score ≤ 0.452) [d]CADD: Combined Annotation Dependent Depletion (phred = scaled CADD-score; CADD-PHRED score of 10 means 10% most deleterious variants, 20 = 1% most deleterious, 30 = 0.1% most deleterious, etc.)



eFigure 1. Distribution of Phenotypic Risk Scores in Eyes of Rare Pathogenic CFH Variant Carriers (green) and Noncarriers (blue). The x-axis represents the phenotypic risk score and the y-axis frequency as percentages within each study group.



eFigure 2. Receiver Operating Characteristic Curve of the Phenotypic Risk Score. The optimal cut-off for this phenotypic risk score is 3 with a sensitivity of 0.86 and specificity of 0.63.

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Eveline Kersten
Maartje J. Geerlings
Jordi Corominas
Marc Pauper
Lebriz Altay
Sascha Fauser
Eiko K. de Jong
Caroline C.W. Klaver
Carel B. Hoyng
Anneke I. den Hollander

In preparation

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GENETIC SCREENING FOR MACULAR DYSTROPHIES IN PATIENTS CLINICALLY DIAGNOSED WITH DRY AGE-RELATED MACULAR DEGENERATION

ABSTRACT

Purpose: It can be clinically challenging to distinguish dry age-related macular degeneration (AMD) from AMD-mimicking dystrophies, and sometimes misdiagnosis occurs. With upcoming therapies for dry AMD it is important to exclude patients with a different retinal disease from clinical trials. Here, we aim to evaluate the occurrence of AMD-mimicking dystrophies in an AMD cohort.

Methods: Whole-exome sequencing (WES) was performed in patients with intermediate AMD or geographic atrophy secondary to AMD (n=218), selected from the European Genetic Database (EUGENDA). Additionally, WES was performed in 133 EUGENDA controls. We analyzed WES data for variants in 19 genes associated with autosomal dominant and recessive macular dystrophies mimicking AMD.

Results: In three cases we identified a pathogenic heterozygous variant (*PRPH2* c.424C>T; p.Arg142Trp) causal for autosomal dominant central areolar choroidal dystrophy (CACD). Phenotypically, these patients all presented with geographic atrophy. In 12 cases we identified a heterozygous variant of unknown clinical significance, but predicted to be highly deleterious, in genes previously associated with autosomal dominant macular dystrophies. One case carried two heterozygous variants in the *IMPG1* gene of unknown clinical significance.

Conclusions: For inclusion in clinical trials it is crucial to select patients who will most likely benefit from these therapies. In our AMD cohort we identified three cases with CACD. The distinction between AMD and AMD-mimicking dystrophies, such as CACD, can be challenging based on phenotype alone. Genetic screening for genes associated with macular dystrophies, especially *PRPH2,* might be beneficial to help identify AMD-mimicking dystrophies.

INTRODUCTION

Age-related macular degeneration (AMD) is a common progressive retinal disorder affecting the elderly.[1] The early stages of AMD are characterized by drusen accumulation in the macula, and as disease progresses two types of advanced AMD can be distinguished: geographic atrophy (GA) and choroidal neovascularization (CNV).[2] Currently, no curative treatment exists for the early and atrophic stages of AMD, which affect the majority of patients (80-90%).[3,4] However, therapies targeting AMD disease pathways are in development and are being evaluated in clinical trials.[3,4] A number of these trials are targeted against components of the complement system. From genetic, physiological and histopathological studies we know that increased activation of the complement system seems to play a major role in AMD pathogenesis.[5-9] To date, results of two phase 2 trials with complement inhibiting agents have been published. Administration of eculizumab did not show a beneficial effect on atrophy progression in the COMPLETE study,[10] while the MAHALO study reported promising results for lampalizumab in reducing atrophy progression.[11] Therapeutic agents targeting other pathways, such as the visual cycle and oxidative stress, are also being evaluated in clinical trials, but thus far without promising results.[4]

In order for clinical trials to be successful, it is crucial to select patients that will most likely benefit from the treatment. Currently, in- and exclusion criteria of clinical trials are based on a clinical diagnosis and are often described in terms of "well demarcated area of GA secondary to AMD" and "exclude GA due to other diseases". Sometimes it is, however, clinically challenging to distinguish AMD from macular dystrophies that are characterized by drusen-like yellowish spots in the macula and geographic atrophy.[12] For example, the early stages of central areolar choroidal dystrophy (CACD) are characterized by pigmentary changes mimicking early AMD,[13] and late-onset Stargardt disease is characterized by the appearance of yellowish flecks in the macula.[14] Both diseases eventually progress to chorioretinal atrophy similar to advanced atrophic AMD. Additionally, several other monogenic macular disorders can be labeled as so-called AMD-mimicking dystrophies.[12] Especially when a patient presents at older age and GA has already developed, it can be challenging to distinguish AMD from GA secondary to other retinal diseases and potentially patients might be misdiagnosed. Before inclusion of patients in clinical trials for dry AMD, it may therefore be useful to perform genetic testing to exclude AMD-mimicking dystrophies. In this study, we aim to evaluate the occurrence of genetic variants associated with autosomal dominant or autosomal recessive AMD-mimicking dystrophies in 218 cases diagnosed with dry AMD.

METHODS

Study population

For this study we selected participants enrolled in the European Genetic Database (EUGENDA), a large multicenter database for clinical and molecular analyses of AMD. Patients selected for this study were classified with intermediate AMD or advanced atrophic AMD. Color fundus photographs of both eyes, and if available spectral domain optical coherence tomograms and fluorescein angiograms, were evaluated by two independent reading center graders according to the Cologne Image Reading Center and Laboratory (CIRCL) protocol.[15] Classification of AMD was based on grading of the worst affected eye. Intermediate AMD was defined as the presence of at least one large druse (diameter ≥ 125µm) or 15 intermediate drusen (diameter 63-125µm) in the Early Treatment Diabetic Retinopathy Study (ETDRS) grid. A sharply demarcated round or oval area of RPE depigmentation (diameter ≥ 175µm) with increased visibility of choroidal vessels within the central circle of the ETDRS grid secondary to AMD without signs of CNV was defined as advanced atrophic AMD. In total, 218 AMD cases were included for analysis. For 33 cases one of more family members were included. In total 62 family members were included, of which 40 were diagnosed with AMD, and 22 did not have signs of AMD . Additionally, 133 control individuals aged 65 years and older without signs of AMD were included in this study.

All individuals provided written informed consent for enrollment in EUGENDA. This research was approved by the local ethical committees at the Radboud university medical center and the University Hospital of Cologne and the study adhered to the tenets of the Declaration of Helsinki.

Whole-exome sequencing

Genomic DNA was extracted from venous blood samples using standard procedures. Whole-exome sequencing (WES) was performed for 218 patients classified with intermediate AMD or advanced atrophic AMD, 62 of their family members, and 133 control individuals. WES capture and variant calling was obtained though the Nimblegen SeqCap EZ Exome v2 kit by paired-end sequencing on an Illumina HiSeq sequencer using TruSeq V3 chemistry (Corominas et al, manuscript submitted). For this study, we analyzed WES data for variants in 19 genes associated with autosomal dominant and recessive macular dystrophies mimicking AMD (Table 1). Filtering of the data was performed to select protein-altering (nonsynonymous), nonsense, frameshift or splice-site variants with a minor allele frequency (MAF) ≤1% in the non-Finnish European population reference panel of the Exome Aggregation Consortium (ExAC),[16] and in the Dutch population reference panel of the Genome of the Netherlands (GoNL) consortium.[17] To minimize the number of false positive variants, additional filter criteria included coverage depth of at least 20 reads, 10 variant reads and 20% variation of reads. A variation of reads between 20% and 80% was defined as heterozygous, and all variants with a variation of reads ≥90%

were named homozygous. Thereafter, we searched literature and public archives (ClinVar[18] and LOVD[19]) to determine if a variant is described to be pathogenic or is of unknown clinical significance (including variants with conflicting interpretations of pathogenicity). We explored the deleteriousness of nonsynonymous missense variants of unknown clinical significance using scaled Combined Annotation Dependent Depletion (CADD phred) prediction scores.[20]

RESULTS

In this study, 126 intermediate AMD cases and 90 advanced AMD cases with GA were included. Of these, 80 AMD cases were male (36.7%) and 138 female (63.3%). Their mean age was 72.7 years (SD 10.4). Gender distribution was comparable to the control individuals with 37.8% male individuals. The mean age of the control individuals was higher (79.3±11.5 years), but this is explained by the inclusion criteria for control individuals (≥65 years of age), while there were no age criteria for AMD cases.

In the next sections, we first describe the occurrence of variants associated with autosomal dominant macular dystrophies, and thereafter variants associated with autosomal recessive macular dystrophies in our AMD cohort.

Variants in genes associated with autosomal dominant macular dystrophies

In our case-control cohort, we identified one heterozygous variant previously described as pathogenic in literature or in public archives. It concerns a missense variant in the *PRPH2* gene in three individuals (c.424C>T, p.Arg142Trp), which is reported to be causal for CACD.[21] All three cases presented with geographic atrophy (**Figure 1**). No other known pathogenic variants were identified in the *PRPH2* gene or other genes associated with autosomal dominant AMD-mimicking dystrophies.

Table 1. Genes associated with AMD-mimicking diseases.

Gene	Chr.	Associated disease
Autosomal Dominant		
BEST1	1	Adult-onset foveomacular vitelliform dystrophy (AFVD) Best vitelliform macular dystrophy (BVMD)
C1QTNF5/MFRP	1	Late onset retinal degeneration (LORD)
CTNNA1	5	Butterfly-shaped pigment dystrophy
EFEMP1	2	Malattia Leventinese (ML)/Doyne honeycomb retinal dystrophy
ELOVL4	6	Stargardt-like macular dystrophy (STGD3) Autosomal dominant macular dystrophy
FSCN2	17	Autosomal dominant macular degeneration Autosomal dominant retinitis pigmentosa
GUCA1B	6	Autosomal dominant retinal degeneration
OTX2	14	Autosomal dominant pattern dystrophy
PRDM13	6	North-Carolina macular dystrophy (NCMD)
PRPH2	6	Central areolar choroidal dystrophy Adult-onset foveomacular vitelliform dystrophy Autosomal dominant pattern dystrophy Pseudo-Stargardt pattern dystrophy
RP1L1	8	Autosomal dominant occult macular dystrophy
TIMP3	22	Sorsby fundus dystrophy
Autosomal Recessive		
ABCA4	1	(late-onset) Stargardt disease
ABCC6	16	Pseudoxanthoma elasticum related dystrophy (angioid streaks)
DRAM2	1	Autosomal recessive macular dystrophy
MFSD8	4	Nonsyndromic autosomal recessive macular dystrophy
Autosomal Dominant or Autosomal Recessive		
IMPG1	6	Autosomal dominant benign concentric annular macular dystrophy Autosomal dominant and autosomal recessive vitelliform macular dystrophies
PROM1	4	Autosomal dominant bull's-eye macular dystrophy Autosomal dominant stargardt-like dystrophy Autosomal recessive cone-rod dystrophy

Additionally, 28 variants of unknown clinical significance were identified, which were not identified in the 133 control individuals (**eTable1**). Because of their uncertain significance further evaluation included only those variants leading to a premature nonsense codon, leading to a shift in the open reading frame, or affecting the invariable splice donor or acceptor sites (-1, -2, +1, +2), and nonsynonymous missense variants predicted to be the 1% most deleterious variants in the human genome (CADD score ≥ 20). In 18 cases we identified a variant of unknown clinical significance predicted to be highly deleterious. For 4 of these cases WES data of family members were available. In all four cases, the variants of unknown clinical significance did not segregate with the disease, and were therefore not considered to be pathogenic. The 12 remaining cases carried a variant of unknown clinical significance in the *BEST1, ELOVL4, FSCN2, IMPG1, OTX2, PRDM13, PROM1* or *RP1L1* gene (**Table 2**). All 12 cases had typical characteristics of intermediate AMD or GA (**Table 2**).

Variants in genes associated with autosomal recessive retinal dystrophies

None of our cases carried homozygous coding variants in genes associated with autosomal recessive macular dystrophies. We identified one case with two heterozygous variants in *IMPG1* (c.336TC>C; p.Ile112Ile*, c.173G>A; p.Arg58Lys) of unknown clinical significance. Additionally, 13 out of 218 cases (6.0%) carried a single heterozygous variant, previously described to be pathogenic in literature or in public archives, in the *ABCA4, ABCC6, MFSD8* or *PROM1* gene (**Table 3**). The allele frequencies of these 8 variants were comparable to MAFs in population reference panels, however, 7 variants were not detected in the 133 control individuals.

DISCUSSION

Phenotypic similarities between macular dystrophies and AMD have been described in literature.[22,12,13] In this study, we evaluated the occurrence of genetic variants associated with autosomal dominant and autosomal recessive AMD-mimicking dystrophies in cases clinically diagnosed with dry AMD.

Table 2. Variants of unknown clinical significance in autosomal dominant macular dystrophy genes identified in cases diagnosed with dry AMD .

	ExAC MAF (%)	**Cases** n (MAF[%])	**Characteristics on retinal imaging of cases carrying a variant of unknown clinical significance**
BEST 1			
c.1193C>T; p.Ser398Phe	0.08	1 (0.23%)	Central geographic atrophy (GA) surrounded by small hard drusen extending to the periphery
ELOVL4			
c.145A>G; p.Thr49Ala	-	1 (0.23%)	Large soft drusen throughout the macula
FSCN2			
c.1057G>A; p.Val353Met	0.04	1 (0.23%)	Reticular pseudodrusen and some soft drusen
IMPG1			
c.1982G>A; p.Arg661His	-	1 (0.23%)	Multifocal GA and some intermediate drusen
c.1945C>T; p.Leu649Phe	0.40	1 (0.23%)	Few intermediate to large soft macular drusen
c.1738C>T; p.Arg580Cys	0.02	1 (0.23%)	Intermediate to large soft macular drusen
c.336TC>C; p.Ile112Ile*	-	1 (0.23%)	Intermediate to large soft macular drusen
OTX2			
c.844T>A; p.Cys282Ser	0.003	1 (0.23%)	Extensive large soft drusen and calcified drusen throughout the macula and reticular pseudodrusen around the retinal arcades
PRDM13			
c.113C>T; p.Ser38Leu	0.07	1 (0.23%)	Numerous small hard (cuticular) drusen throughout the macula extending beyond the vascular arches
PROM1			
c.1345G>A; p.Val449Met	0.20	1 (0.23%)	Central GA surrounded by intermediate to large drusen and some peripheral drusen
c.155T>C; p.Ile52Thr	0.003	1 (0.23%)	Drusen deposition throughout the macula
RP1L1			
c.553G>T; p.Ala185Ser	-	1 (0.23%)	Multifocal GA surrounded by large soft drusen

Variants leading to a premature nonsense codon, frameshift, affecting the splice donor or acceptor sites (-1, -2, +1, +2), and nonsynonymous missense variants predicted to be the 1% most deleterious variants in the human genome (CADD score ≥ 20).

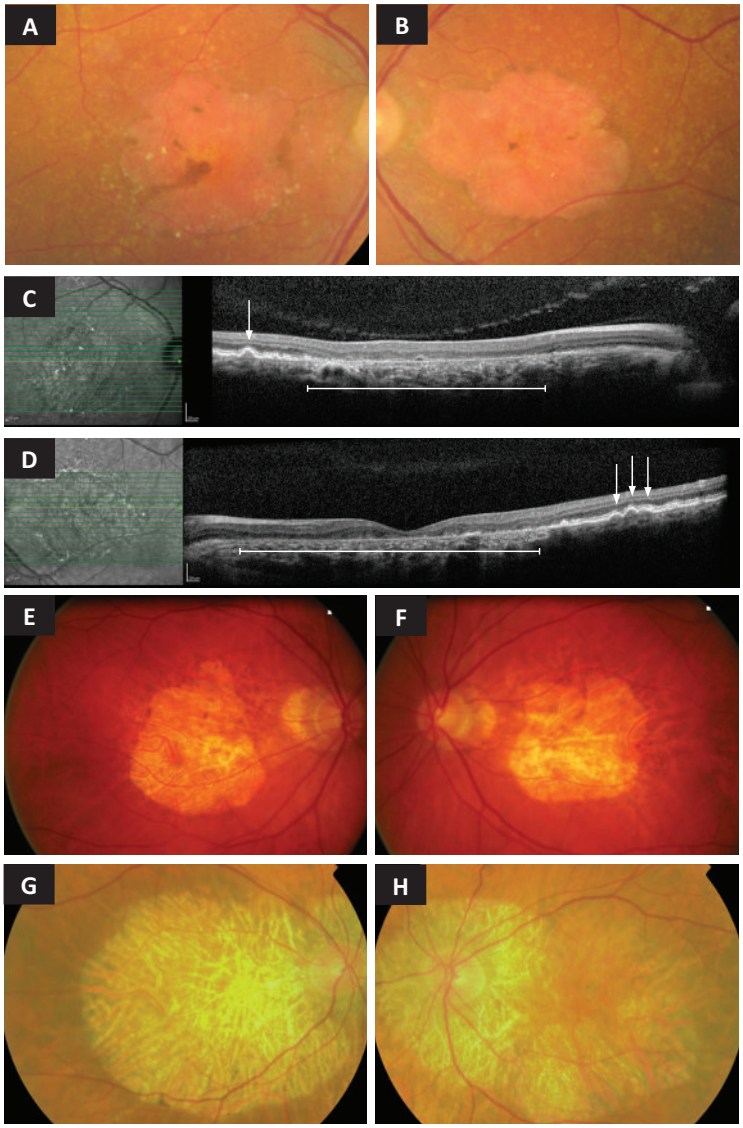

Figure 1. Retinal images of three patients with geographic atrophy secondary to autosomal dominant central areolar choroidal dystrophy (CACD) caused by a heterozygous variant in *PRPH2* (c.424C>T, p.Arg142Trp). Patient 1 (A-D). Color fundus photographs of right (A) and left (B) eye of a 67-year-old female with geographic atrophy and foveal sparing surrounded by drusen secondary to CACD. On the Optical Coherence Tomography (OCT) images of both eyes (C+D) drusen are visible near the edges of the central atrophy. Drusen are indicated by arrows, atrophy is indicated by a continuous line with dashes just below the atrophic area. Patient 2 (E-F). A 64-year-old male with central atrophy in both eyes secondary to CACD. Patient 3 (G-H). A 76-year-old male with extensive geographic atrophy and peripapillary atrophy in both eyes.

Table 3. Variants in autosomal recessive macular dystrophy genes previously described as pathogenic.

	ExAC MAF (%)	**GoNL** MAF (%)	**Cases** total n=218 n(MAF[%])	**Controls** total n=133 n(MAF[%])	**Disease association**
ABCA4					
c.6089G>A; p.Arg2030Gln	0.06%	0.10%	1 (0.23%)	-	Stargardt disease[58]
c.3113C>T; p.Ala1038Val	0.20%	0.30%	1 (0.23%)	-	Stargardt disease[58]
c.2947A>G; p.Thr983Ala	-	-	1 (0.23%)	-	Stargardt disease[58]
c.2588G>C; p.Gly863Ala	0.81%	0.80%	4 (0.92%)	1 (0.38%)	Stargardt disease[58]
c.2546T>C; p.Val849Ala	0.01%	-	1 (0.23%)	-	Stargardt disease[58]
ABCC6					
c.2787+1G>T; p.?	0.02%	-	2 (0.46%)	-	Pseudoxanthoma elasticum[59,60]
MSFD8					
c.1006G>C; p.Glu336Gln	0.33%	0.30%	2 (0.46%)	-	Nonsyndromic autosomal recessive macular dystrophy[61]
PROM1					
c.1355A>TA; p.Tyr452Tyr*	0.03%	-	1 (0.23%)	-	Autosomal recessive cone-rod dystrophy[42]

Age-related macular degeneration and central areolar choroidal dystrophy

We identified three patients carrying a heterozygous variant in the *PRPH2* gene (p.Arg142Trp). This variant causes a central cone dystrophy phenotype associated with autosomal dominant CACD.[23,21]. There are strong phenotypic similarities between CACD and AMD.[13] Stage 1 and 2 of CACD are characterized by focal parafoveal RPE changes and a mottled autofluorescence pattern.[24,13] Additionally, CACD has been described in combination with the presence of drusen in some families.[25] These phenotypes can very much resemble the RPE alterations and drusen as present in early AMD, and like in early AMD, patients often do not have visual complaints at this stage.[24] As CACD progresses, parafoveal atrophy of the retinal pigment epithelium and choriocapillaris develops (stage 3), eventually also involving the fovea (stage 4).[24,13] Most patients with advanced stage CACD are aged between 40 and 70 years old and present with vision loss and central scotomas.[24] An overlap in age of onset of CACD with atrophic AMD was also described by Boon and colleagues.[23] Furthermore, CACD can be easily overlooked and misdiagnosed with AMD based on ophthalmological examination alone, especially in families with incomplete penetrance, which may mask the autosomal dominant inheritance of CACD. Additional imaging, such as spectral-domain optical coherence tomography and fundus autofluorescence imaging, or genetic analyses could help distinguishing between these two diseases.[13]

Different heterozygous variants in the *PRPH2* gene have been described to be causal for autosomal dominant CACD.[23,26,21,27,28] In this study, we detected the p.Arg142Trp variant while no other variants in the *PRPH2* gene were found. Family studies strongly suggest that this variant represents a founder mutation with its origin in the southeast of the Netherlands.[23] This could explain why we detected this specific *PRPH2* variant and no other variants in *PRPH2*. In different regions however, other variants could be more prevalent. For example, a higher frequency of the p.Arg172Trp variant has been described in British patients, also due to a founder effect.[29] Additionally, a splice-site variant in *PRPH2* (c.828+3A>T) is also considered a founder mutation and has been associated with diverse retinal phenotypes besides CACD.[30] Considering the fact that variant frequencies differ between populations, it is to be advised to sequence the entire coding region of the *PRPH2* gene for variants, rather than screening for a specific variant.

Autosomal dominant macular dystrophies mimicking AMD

In this study we also report several variants in genes associated with autosomal dominant and recessive macular dystrophies of unknown clinical significance. We describe 12 variants that are predicted to be deleterious in genes associated with autosomal dominant macular dystrophies (*BEST1, ELOVL4, FSCN2, IMPG1, OTX2, PRDM13, PROM1* and *RP1L1*).

To date, several variants in the *ELOVL4* gene, most frequently a 5-bp deletion in exon 6, have been associated with Stargardt-like macular dystrophy (*STGD3*). This study describes a missense variant in exon 2, in contrast to previously described variants, which are located at the 3' end of the gene. Therefore, it is unlikely that the p.Thr49Ala variant in *ELOVL4* gene identified in one AMD patient in this study is disease-causing. Also, evaluation of the *ELOVL4* gene in AMD patients showed no association with AMD in previous studies.[31,32]

The *FSCN2* gene is described to be a candidate gene for autosomal dominant macular degeneration (RetNet, the Retinal Information Network). One variant (c.208delG) is reported to be associated with the disease in Japanese patients,[33] but this could not be confirmed in Chinese and Spanish studies.[34,35] Other variants in *FSCN2* have been described, but lacked co-segregation.[34] The association of this gene with autosomal dominant macular dystrophy is therefore unclear. It is therefore unlikely that the missense variant p.Val353Met in *FSCN2* identified in one AMD patient in this study is disease-causing.

In autosomal dominant North-Carolina Macular Dystrophy the disease-causing variants are located in the *PRDM13* gene. A few missense variants and tandem duplications have been described.[36,37] We can therefore not exclude that the p.Pro38Leu in *PRDM13* identified in one AMD patient in this study is disease-causing.

Variants in the *IMPG1* gene can cause both autosomal dominant and recessive vitelliform macular dystrophies, and one heterozygous missense variant in *IMPG1* has been described in dominant benign concentric annular macular dystrophy.[38,39] Four AMD patients in this study were found to carry a potentially pathogenic variant in the *IMPG1* gene, and we cannot exclude that these variants may be disease-causing.

Variants in *PROM1* have mainly been associated with autosomal recessive cone-rod dystrophies, a severe retinal phenotype with early onset.[40,41] However, associations between this locus and autosomal dominant macular dystrophies have also been described.[42,43] We cannot therefore not exclude that the *PROM1* p.Ile52Thr and p.Val449Met variants identified in two AMD patients in this study may be disease-causing.

OTX2 variants can cause severe ocular disorders, such as developmental ocular malformations or Leber congenital amourosis, but a heterozygous missense variant (p.Glu79Lys) was reported in two families with autosomal dominant pattern dystrophy, implicating the involvement of *OTX2* in pattern dystrophies.[44] A different missense variant, p.Cys282Ser, was identified in *OTX2* in one patient in this study, and we cannot exclude that this variant may be disease-causing.

Several heterozygous missense variants in *RP1L1* have been associated with occult macular dystrophy, mainly in Asian families.[45-47] Variant screening in the *RP1L1* gene is complicated because the gene is highly polymorphic and shows a high degree of similarity with *RP1*,[48] therefore replication of the variant described in this study is warranted.

Various variants in the *BEST1* gene have been reported to be associated with autosomal dominant vitelliform macular dystrophies.[49] Most of these variants are missense mutations and located within the first eight exons, whereas the p.Ser398Phe variant we report here is located in exon 9 of the gene. It is therefore unlikely that the p.Ser398Phe variant in *BEST1* identified in one AMD patient in this study is disease-causing.

Summarizing, we identified 12 potentially deleterious variants but of yet unknown clinical significance in genes associated with autosomal dominant macular dystrophies. It is unlikely that the variants in the *BEST1, ELOVL4* and *FCSN2* genes are disease-causing, but we cannot rule out the possibility that the variants in the *PRDM13, IMPG1, PROM1, OTX2* and *RP1L1* genes might be disease-causing. Therefore one might consider to exclude patients carrying variants in genes associated with autosomal dominant macular dystrophies from clinical trials, in particular is the disease phenotype matches previously reported disease characteristics of these dystrophies. Other studies are needed to elucidate the potential pathogenicity of these novel variants.

Susceptibility of development of age-related macular degeneration in heterozygous carriers of autosomal recessive macular dystrophies

Besides phenotypic similarities between AMD and macular dystrophies, it has been suggested that carriers of a single *ABCA4* variant are at increased risk of developing AMD compared to noncarriers.[50,51] In this study, we identified seven cases that carried a heterozygous *ABCA4* variant previously reported to be pathogenic, compared to only one control individual carrying a heterozygous pathogenic *ABCA4* variant. However, the number of control individuals in this study was too small to draw conclusions. Larger studies are needed to evaluate the hypothesis that carriers of heterozygous variants associated with autosomal recessive macular dystrophies might be at increased risk for AMD development.

Clinical implications

It is increasingly important to correctly diagnose patients with macular degeneration with respect to inclusion in clinical trials and for future treatment. For both dry AMD and macular dystrophies no curative treatment is available in clinical routine yet, though multiple clinical trials are ongoing.[4] To achieve a favorable outcome of clinical trials, selection of those patients who will most likely benefit from the treatment is essential. While treatment is still in development, for some macular diseases lifestyle interventions are recommended, including dietary changes or nutritional supplementation. Results of the Age-Related Eye Disease Study 2 (AREDS2) indicate that oral supplementation of antioxidants (lutein, zeaxanthin, vitamins C and E) and zinc reduces the risk of progression to advanced AMD in patients with nonadvanced AMD or unilateral advanced AMD.[52] Furthermore, high dietary intake of antioxidants and fish consumption can reduce the risk of advanced AMD among people with a high genetic risk of AMD.[53] Contrarily, patients with Stargardt disease are recommended to limit vitamin A and beta-carotene intake, as they cannot metabolize vitamin A properly due to ABCA4 protein impairment.[54] Low dietary intake of vitamin A in patients with Stargardt disease has been associated with better visual acuity compared to those with higher vitamin A intake.[55] These different nutritional interventions between macular diseases underline the importance of proper diagnosis of AMD and AMD-mimicking dystrophies.

Concluding, for clinical trials and future therapies for AMD it is important to identify those patients that will benefit most likely from the treatment and exclude AMD-mimicking dystrophies. Detailed phenotyping is necessary for distinguishing different macular diseases, and multimodal imaging can be useful. Despite modern imaging technologies, however, it can be difficult to clinically differentiate AMD from AMD-mimicking dystrophies. Genetic screening of genes involved in AMD-mimicking dystrophies can aid in establishing an accurate diagnosis. Based on the findings of this study, genetic screening of the *PRPH2* gene is recommended because of the significant clinical overlap between CACD and AMD.

SUPPLEMENTARY INFORMATION

Gene name	Inheri-tance	chr	Start position	Ref	Alt	c.DNA change	Protein change	ExAC NFE	AF_GoNL	CADD_ phred	reads	Variation reads	% Variation	Cases (n=218)	Controls (n=133)
ABCA4	AR	1	94467548	C	G	c.6148G>C	V2050L	0.0037	0.201	33	36	16	44.44	1	0
ABCA4	AR	1	94473287	G	A	c.5908C>T	L1970F	0.0042	0.502	26.4	42.33	20.67	48.83	1	2
ABCA4	AR	1	94476377	C	T	c.5693G>A	R1898H	0.0028	0.1	22.8	48.5	21	43.3	1	1
ABCA4	AR	1	94476874	C	T	c.5528G>A	R1843Q	1.50E-05	0	35	35	14	40	1	0
ABCA4	AR	1	94480221	G	C	c.5338C>G	P1780A	0.0002	0	26.2	54	35	64.81	1	0
ABCA4	AR	1	94487404	C	T	c.4771G>A	G1591R	0.0045	0.402	24.6	77.75	36.25	46.62	1	3
ABCA4	AR	1	94496039	C	T	c.4297G>A	V1433I	0.0019	0.502	20.9	31	13.5	43.55	1	0
ABCA4	AR	1	94506773	C	T	c.3514G>A	G1172S	1.50E-05	0	9.41	29	12	41.38	1	0
ABCA4	AR	1	94514466	T	C	c.2701A>G	T901A	0.0027	0.201	9.516	85	45	52.94	1	0
ABCA4	AR	1	94514477	G	A	c.2690C>T	T897I	0.0018	0.402	23.6	83	46	55.42	1	0
ABCA4	AR	1	94528142	A	C	c.1928T>G	V643G	0.0022	0.703	27.5	30.75	15.25	49.59	2	2
ABCA4	AR	1	94528774	C	T	c.1654G>A	V552I	0.0039	0.602	15.19	37.67	19	50.44	1	2
ABCA4	AR	1	94528818	C	T	c.1610G>A	R537H	0.0024	0.301	26.2	37	21	56.76	1	0
ABCA4	AR	1	94546094	C	A	c.1039G>T	A347S	.	0	25.4	152	67	44.08	1	0
ABCA4	AR	1	94568627	C	T	c.514G>A	G172S	0.0006	0	23.4	154	73	47.4	1	0
ABCA4	AR	1	94568675	T	C	c.466A>G	I156V	0.0017	0.1	5.706	162.5	70	43.08	2	2
ABCA4	AR	1	94568686	C	T	c.455G>A	R152Q	0.0031	0.402	21.5	158	78	49.37	1	0
ABCA4	AR	1	94574244	C	T	c.331G>A	E111K	.	0	21.4	74	38	51.35	1	0
ABCC6	AR	16	16271467	G	A	c.2432C>T	T811M	0	0	28.5	35	16	45.71	1	0
ABCC6	AR	16	16282693	C	T	c.1774G>A	V592I	1.73E-05	0	11.72	20	12	60	1	0
ABCC6	AR	16	16286695	G	C	c.1423C>G	H475D	1.52E-05	0	0.323	43	19	44.19	1	0
ABCC6	AR	16	16286750	G	C	c.1368C>G	I456M	0.0002	0	12.35	46.8	21	44.87	2	2
ABCC6	AR	16	16291993	T	A	c.1223A>T	D408V	.	0	23	20	10	50	1	0

BEST1	AD	11	61729819	C	T	c.1013C>T	S338F	0.0008	0.1	27.8	31	19	61.29	1	0
BEST1	AD	11	61730145	T	C	c.1339T>C	S447P	0.0018	0	2.919	47	19	40.43	1	0
CTNNA1	AD	5	138147939	C	T	c.536C>T	A179V	0.0018	0.301	23.2	270.25	126.5	46.81	3	0
CTNNA1	AD	5	138269759	A	G	c.2702A>G	K901R	.	0	15.99	43	16	37.21	1	0
EFEMP1	AD	2	56145171	T	G	c.146A>C	D49A	0.0013	0.301	15.49	28.8	13.4	46.53	4	1
ELOVL4	AD	6	80626470	A	G	c.800T>C	I267T	0.0068	0.904	8.833	102	49.72	48.75	5	2
ELOVL4	AD	6	80636054	T	C	c.145A>G	T49A	.	0	24.3	100	42	42	1	0
FSCN2	AD	17	79503213	G	A	c.1025G>A	R342Q	0.0002	0	23	49.5	28	56.57	1	0
FSCN2	AD	17	79503245	G	A	c.1057G>A	V353M	0.0004	0.602	27.9	36	15	41.67	1	0
IMPG1	AD/AR	6	76640695	G	A	c.2218C>T	L740F	0.0007	0.1	10.89	28	16	57.14	1	0
IMPG1	AD/AR	6	76640830	C	T	c.2083G>A	E695K	.	0	15.38	39	21	53.85	1	0
IMPG1	AD/AR	6	76657093	C	T	c.1982G>A	R661H	0	0	23.5	66	33	50	1	0
IMPG1	AD/AR	6	76657130	G	A	c.1945C>T	L649F	0.004	0.402	27.4	106	52	49.06	1	0
IMPG1	AD/AR	6	76660365	G	A	c.1738C>T	R580C	0.0002	0	34	21	12	57.14	1	0
IMPG1	AD/AR	6	76660397	T	C	c.1706A>G	K569R	0.0039	0.301	15.88	38.4	18.6	48.44	2	0
IMPG1	AD/AR	6	76731868	C	T	c.631G>A	D211N	0.0008	0	3.873	139	64	46.04	1	1
IMPG1	AD/AR	6	76744470	GA	G	c.336TC>C	I112IX	.	0	.	31	10	32.26	1	0
IMPG1	AD/AR	6	76751738	C	T	c.173G>A	R58K	0.0055	0.301	10.32	123.5	55.5	44.94	2	0
MFRP	AD	11	119214636	G	T	c.1014C>A	S338R	0.0015	0	7.075	25	11.5	46	1	1
MFRP	AD	11	119216274	G	C	c.497C>G	P166R	1.51E-05	0	23.6	20	10	50	1	1
MFSD8	AR	4	128842876	C	G	c.1153G>C	G385R	0.0005	0	6.644	138	58	42.03	1	0
MFSD8	AR	4	128842893	A	G	c.1136T>C	F379S	0.0003	0.201	15.18	137.5	66.5	48.36	1	1
MFSD8	AR	4	128843095	A	G	c.1022T>C	L341P	0	0	27.4	167	68	40.72	1	0
OTX2	AD	14	57268503	A	T	c.844T>A	C282S	3.00E-05	0	24.3	73	33	45.21	1	0
OTX2	AD	14	57268922	G	C	c.425C>G	P142R	0.0002	0	21.3	50	26	52	1	0

Gene name	Inheri-tance	chr	Start position	Ref	Alt	c.DNA change	Protein change	ExAC NFE	AF_GoNL	CADD_ phred	reads	Variation reads	% Variation	Cases (n=218)	Controls (n=133)
PRDM13	AD	6	100055023	C	T	c.113C>T	S38L	0.0007	0	28.8	49	22	44.9	1	0
PROM1	AD/AR	4	15982084	T	C	c.2450A>G	K817R	.	0	31	135	61	45.19	1	0
PROM1	AD/AR	4	15987578	A	G	c.2184+2T>C	?	.	0	18.29	29	18	62.07	1	0
PROM1	AD/AR	4	15992900	G	C	c.1928C>G	A643G	0.001	0.1	8.738	176	78	44.32	1	0
PROM1	AD/AR	4	16008270	C	T	c.1345G>A	V449M	0.002	0.201	20.4	30	11	36.67	1	0
PROM1	AD/AR	4	16040595	A	G	c.250T>C	Y84H	.	0	7.446	98	53	54.08	1	0
PROM1	AD/AR	4	16077375	A	G	c.155T>C	I52T	3.12E-05	0	23.2	58	24	41.38	1	0
PROM1	AD/AR	4	16077475	A	C	c.55T>G	S19A	0.0091	1.004	1.486	44.86	23	51.27	2	3
RP1L1	AD	8	10464616	G	A	c.6992C>T	T2331M	0.0023	0.402	1.738	42.67	19.67	46.1	3	0
RP1L1	AD	8	10465024	C	A	c.6584G>T	G2195V	1.50E-05	0	9.777	23	11	47.83	1	0
RP1L1	AD	8	10467629	T	TTTC	c.3979A>GAAA	T1327E	.	0	.	97.31	45.52	46.78	13	2
RP1L1	AD	8	10467637	T	TCCTCTAA CTGCACC CTCTCTTC TTGCAGC CCTTCTA TTACTTT AGTCC	c.3971A>GGAC TAAAGTAATAG AAGGGCTGCAA GAAGAGAGGGT GCAGTTAGAGGA	E1324G	.	0	.	25.67	21	81.81	1	5
RP1L1	AD	8	10468963	C	T	c.2645G>A	R882Q	0	0	6.663	20	10	50	1	0
RP1L1	AD	8	10480144	G	A	c.568C>T	R190C	0.0027	0.904	17.97	43.4	19.4	44.7	4	1
RP1L1	AD	8	10480159	C	A	c.553G>T	A185S	.	0	26.2	38	21	55.26	1	0

Chr =chromosome; ref = reference

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Maartje J. Geerlings*
Elena B. Volokhina*
Eiko K. de Jong
Nicole van de Kar
Marc Pauper
Carel B. Hoyng
Lambert P. van den Heuvel
Anneke I. den Hollander
*these authors contributed equally to this study

Submitted

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GENOTYPE-PHENOTYPE CORRELATIONS OF LOW FREQUENCY GENETIC VARIANTS IN THE COMPLEMENT SYSTEM IN RENAL DISEASE AND AGE-RELATED MACULAR DEGENERATION

ABSTRACT

Genetic alterations in the complement system have been linked to a variety of diseases, including atypical hemolytic uremic syndrome (aHUS), C3 glomerulopathy (C3G), and age-related macular degeneration (AMD). We performed sequence analysis of the complement genes *CFH*, *CFI*, and *C3* in 866 renal diseases patients (aHUS/C3G) and 697 AMD patients. Genotype-phenotype correlations between the disease groups identified a higher frequency of protein-altering alleles in SCR20 of FH, and in the serine protease domain of FI in aHUS/C3G patients. In AMD a higher frequency of protein-altering alleles was observed in SCR3, SCR5 and SCR7 of FH, the FI SRCR domain, and the MG3 domain of C3. Our data underscore the importance of the N-terminal SCRs of FH in C3b regulation in AMD, and the specificity with which the C-terminal SCRs of FH interact with the glomerular endothelium. Alterations in the SRCR domain of FI are likely to affect expression and thus overall activity, while FI changes related to renal disease are involved in structural elements around the active site. Alterations located in the MG3 domain of C3 in AMD patients may hamper regulation of C3. We observed a substantial overlap of variants between aHUS/C3G and AMD, however, there is a distinct clustering of variants within specific domains.

KEY WORDS

Atypical hemolytic uremic syndrome, C3 glomerulopathy, Age-related macular degeneration, Complement system, Alternative pathway

INTRODUCTION

The complement system is part of the innate immune system which balances host protection and immune defense. The complement system can be activated via three pathways: the classical, lectin and alternative pathways, which converge at the step of cleavage of the central component C3 into C3a and C3b. C3b bound to activated factor B (Bb) forms the alternative pathway C3 convertase (C3bBb), which cleaves and activates more C3 molecules, thereby amplifying the cascade. When the C3 convertase binds additional C3b molecules, the C5 convertase is formed (C3bBbC3b), which cleaves C5 into C5a and C5b. C5b interacts with C6, C7, C8 and multiple C9 molecules forming the membrane attack complex (C5b-9). Regulators, such as factor H (FH) and membrane cofactor protein (MCP; CD46), can inhibit complement activation by accelerating the decay of the C3 convertases or by acting as a cofactor for factor I (FI).[1]

Deregulation of the complement system, specifically the alternative pathway, has been implicated in a variety of diseases. Remarkably, protein-altering variants in genes of the complement system have been associated with very different clinical outcomes: atypical hemolytic uremic syndrome (aHUS; MIM# 235400), C3 glomerulopathy (C3G; or dense deposit disease [DDD]), and age-related macular degeneration (AMD; MIM# 603075).[2,3]

aHUS is a rare acute disorder characterized by acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia. It is estimated that genetic variants in the *complement factor H (CFH), complement factor I (CFI), complement C3 (C3), complement factor B (CFB), CD46 molecule (CD46), thrombomodulin (THBD), and diacylglycerol kinase epsilon (DGKE)* genes, the presence of genomic rearrangement in the *CFH/CFH-related (CFHR*) gene cluster, and autoantibodies to FH account for 60% of all aHUS cases.[4-6]

C3G is another rare renal illness, which is characterized by C3 deposition in the glomeruli of the kidney and can lead to renal failure. C3G pathogenesis is linked to the presence of autoantibodies that stabilize the alternative pathway and classical pathway C3 convertases (C3Nef and C4Nef). Genetic aberrations in the *CFH, CFI, C3, CD46, CFHR5* genes, genomic rearrangements in the *CFH/CFHR* gene cluster, and anti-FH autoantibodies genes have been described in 20% of C3G patients.[6-8]

AMD, in contrast to aHUS and C3G, is a common disease in which gradual visual impairment occurs at older age due to degeneration of the central retina. The disease is characterized by the disruption of normal retinal pigment epithelium (RPE) function through the accumulation of waste products, called drusen, between Bruch's membrane and the RPE. A combination of multiple genetic and environmental factors contribute to the pathogenesis of AMD. More than

a third of the disease-associated genetic variants reside in or near genes of the complement system: *CFH, CFI, C3, complement C2 (C2)/CFB, complement C9 (C9)*, and *vitronectin (VTN)*.[9] In addition, rare protein-altering variants in the *CFH*, *CFI, C3* and *C9* genes have been associated with AMD.[9,10].

A number of low frequency protein-altering variants have been described to cause both aHUS/C3G and AMD, such as the p.Arg53His and p.Arg1210Cys variants in *CFH,*[11,12] p.Gly119Arg and p.Gly287Arg in *CFI*[13,14] and p.Lys155Gln and p.Arg161Trp in *C3*.[10,15,16] A pathophysiologic explanation on how the same mutation, in different patients, can lead to a different disease is not available.[1] It has been suggested that the final disease outcome is determined by the individual's overall genetic risk to each of these diseases, and is influenced by environmental factors.[17]

However, a clustering of variants in certain protein domains in the different diseases has been described, supporting the existence of a genotype-phenotype correlation.[18]. In C3, the p.Arg161Trp variant is located in the MG2 domain and is present in 4-16% of aHUS patients.[19,20] A particularly prominent hotspot for aHUS mutations is located within the C-terminal short consensus repeat (SCR) (15-20) domains of FH, and is considered typical for this disease. Such clustering of variants in FH was not observed for C3G patients.[21] In AMD, an enrichment of protein-altering variants has been reported in the N-terminal and C-terminal SCR domains of FH, and in the serine protease domain of FI.[1,22,23] However, a systematic evaluation and comparison of genetic aberrations between the renal diseases (aHUS/C3G) and AMD has not yet been performed. Such analysis could provide a more in depth insight into genotype-phenotype correlations for these diseases, and could considerably enhance our understanding of complement deregulation in human disease.

In this study, we describe genetic variants identified in the *CFH, CFI,* and *C3* genes in large patient cohorts consisting of 866 renal disease (aHUS/C3G) patients and 697 AMD patients, and provide a comprehensive genotype-phenotype correlation analyses between these disease groups.

MATERIALS AND METHODS

Cohort description

The AMD cohort consisted of 697 individuals who were recruited as part of the European Genetic Database (EUGENDA) between December 2005 and June 2014. Only individuals affected by AMD were included in this study. To diagnose AMD, retinal images were evaluated according to the Cologne Image Reading Center (CIRCL) protocol. In short, AMD was characterized

as the presence of pigmentary changes together with at least 10 small drusen (<63 μm) or the presence of intermediate (63-124 μm) to large drusen (≥125 μm diameter) near the macula. Furthermore, late AMD was defined as either subfoveal geographic atrophy (GA) or choroidal neovascularization (CNV) in at least one eye. Written informed consent was obtained from all participants. The study was approved by the local ethics committees on Research Involving Human Subjects, and conducted according to the Declaration of Helsinki.

The aHUS/C3G cohort consisted of 886 patients that were referred to the Radboud university medical center for genetic screening between 2007 and 2015 to confirm the clinical diagnosis of aHUS or C3 glomerulopathy. The diagnosis of aHUS was defined as a presence of hemolytic anemia, thrombocytopenia and acute renal failure, that was not preceded with infection with Shiga toxin producing *E. coli*.[6] C3G diagnosis was defined as active glomerulonephritis combined with predominantly C3 absent or marginal immunoglobulin deposition in renal biopsy [24]. Most of the patients (around 70%) in this cohort were diagnosed with aHUS. However, in some of the cases the clinical diagnosis of aHUS or C3G was unclear, and therefore a genetic screening was requested as part of their clinical care..

Genetic screening

In this study we focused on the complement genes associated with both aHUS/C3G and AMD, which includes the *CFH* (HGNC:4883, MIM# 134370), *CFI* (HGNC:5394, MIM# 217030), and *C3* (HGNC:1318, MIM# 120700) genes [4,5,9,22].

Genetic analysis was performed for the aHUS/C3G and AMD cohorts using DNA isolated from peripheral blood leucocytes using standard procedures. For the AMD cohort, whole-exome sequencing (WES) of 697 unrelated individuals was performed. WES capture and variant calling was obtained through the Nimblegen SeqCap EZ Exome v2 kit by paired-end sequencing on a Illumina HiSeq sequencer using TruSeq V3 chemistry as described in detail elsewhere (Corominas et al, manuscript submitted). WES data of the AMD cohort was filtered to select coding non-synonymous and canonical splice-site variants in *CFH (*NM_00186.3), *CFI* (NM_00204.3), and *C3* (NM_000064.3). The mean coverage (and minimum to maximum range) for *CFH, CFI,* and *C3* were 135X (32-495), 124X (44-356), and 32X (2-137), respectively (**Figure S1**).

Strict quality filters were set to obtain true positive hits including minimum read depth (n>20), variant reads (>10) and at least 20% should be variation reads. Variants identified in *CFH* were confirmed by Sanger sequencing due to the high similarities between *CFH* and the *CHFR1-4* genes. For the aHUS/C3G cohort, genetic analysis was performed by amplification of the coding regions and splice junctions of the *CFH* (NM_00186.3), *CFI* (NM_00204.3), and *C3* (NM_000064.3) by PCR, followed by Sanger sequencing or next-generation sequencing on an Ion torrent semiconductor (minimum coverage 40X).

Only low frequency and rare variants (minor allele frequency <5%) based on the ExAC database[25] were taken into account. Variants were considered aHUS/C3G- or AMD-specific when found only in the aHUS/C3G or AMD cohorts analyzed in this study and in literature. Reported odds-ratios for genetic variants in AMD were recently published by the International AMD Genomics Consortium.[9,10] Annotation of the variants, including minor allele frequency of the ExAC database, were obtained using ANNOVAR.[26]

Statistical analysis

Statistical analyses were done using SPSS Software for Windows version 22.0 (Fisher exact, 2-sided, weighted by number). We calculated if there was a higher percentage of alleles within a specific protein domain of FH, FI, or C3 in aHUS/C3G compared to AMD, and vice versa. P-values <0.05 were considered as significant.

Protein structure analysis

Protein domains that carried a significantly higher percentage of alleles in aHUS/C3G or AMD were analyzed in more depth to determine the potential effect of the variants mapping to these domains. Variants were mapped on the available molecular structures of the C3b-miniFH-FI protein complex (PDB 5O32 [27]). Protein structures were retrieved from the protein data bank (PDB, www.rcsb.org)[28] using YASARA.[29] .

Literature search

We performed a literature search to review low frequency coding non-synonymous and canonical splice-site variants in *CFH, CFI*, and *C3* previously reported in aHUS, C3G and AMD. Moreover, variants were extracted from the Factor H aHUS Mutation Database (http://www.fh-hus.org/), and the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/).

RESULTS

Genetic variants identified in aHUS/C3G and AMD patients

We screened 886 aHUS/C3G patients and 697 AMD patients for low frequency variants in the coding and splice-site regions of *CFH*, *CFI*, and *C3*. In total, we identified 505 low frequency alleles in *CFH, CFI*, and *C3*, of which 379 were found in aHUS/C3G and 126 were found in AMD. In aHUS/C3G patients, we identified 225 heterozygous and 7 homozygous carriers of variants in *CFH*, 46 heterozygous and 2 homozygous carriers of variants in *CFI*, and 88 heterozygous carriers and 1 homozygous carrier of variants in *C3*. All 126 variants identified in AMD patients were heterozygous, of which 55 were found in *CFH*, 31 in *CFI* and 40 in *C3*. **(Table S1)**.

The 505 alleles represent 121 unique low frequency variants, of which 51 are novel and 70 were previously reported in literature (**Table S2**). Of the 51 novel variants identified, 8 variants in *CFH* (n=4), *CFI* (n=2), and *C3* (n=2) lead to a premature stop, frameshift or abolished splice site, thereby leading to loss of protein function. The remaining 43 novel variants lead to missense alterations or to an in-frame insertion (**Table S3**).

CFH contained the largest number of unique low frequency variants (n=64). Two-third of these variants were aHUS/C3G- (24/64) or AMD-specific (17/64), and one third was found in both patients groups (23/64). (**Figure 1; Table S3**). Three of the low frequency variants (p.Gln950His, p.Asn1050Tyr, p.Gln1143Glu) identified in *CFH* in aHUS/C3G patients, were previously reported to have a protective effect for AMD.[10] The p.Gln950His variant was detected in 15 aHUS/C3G patients; the p.Asn1050Tyr variant was detected in 21 aHUS/C3G patients; and the p.Gln1143Glu variant was detected in 9 aHUS/C3G patients

In *CFI*, a total of 25 unique low frequency variants was identified of which the majority were found in both patient groups (15/25). Only 4 (4/25) variants were specific for aHUS/C3G and 6 (6/25) variants were exclusively found in AMD (**Figure 1; Table S3**).

A total of 32 unique low frequency variants was identified in *C3*. These variants were evenly distributed between aHUS/C3G-specific variants (13/32), AMD-specific variants (9/32) and both phenotypes (10/32) (**Figure 1; Table S3**).

Genotype-phenotype correlations in aHUS/C3G and AMD

To determine genotype-phenotype correlations, we calculated if there was a higher percentage of alleles within a specific protein domain of FH, FI, or C3 in aHUS/C3G compared to AMD, and vice versa. We observed a higher frequency of protein-altering alleles in the SCR20 domain of FH and in the serine protease domain of FI in aHUS/C3G compared to AMD. In AMD we observed a higher frequency of protein-altering alleles in the SCR3, SCR5 and SCR7 domains of FH, the SRCR domain of FI, and the MG3 domain of C3 (**Table 1)** compared to aHUS/C3G.

For FH we calculated the cumulative effect for the alleles found in the domains involved in cofactor activity, which include SCR1-4 at the N-terminus and SCR19-20 at the C-terminus of the protein. We observed a burden of alleles in the SCR19-20 domains at the C-terminus for aHUS/C3G (21.3% versus 3.6% in AMD), and a burden in the SCR1-4 domains at the N-terminus for AMD (20.0% versus 4.6% in aHUS/C3G)(**Table S3**).

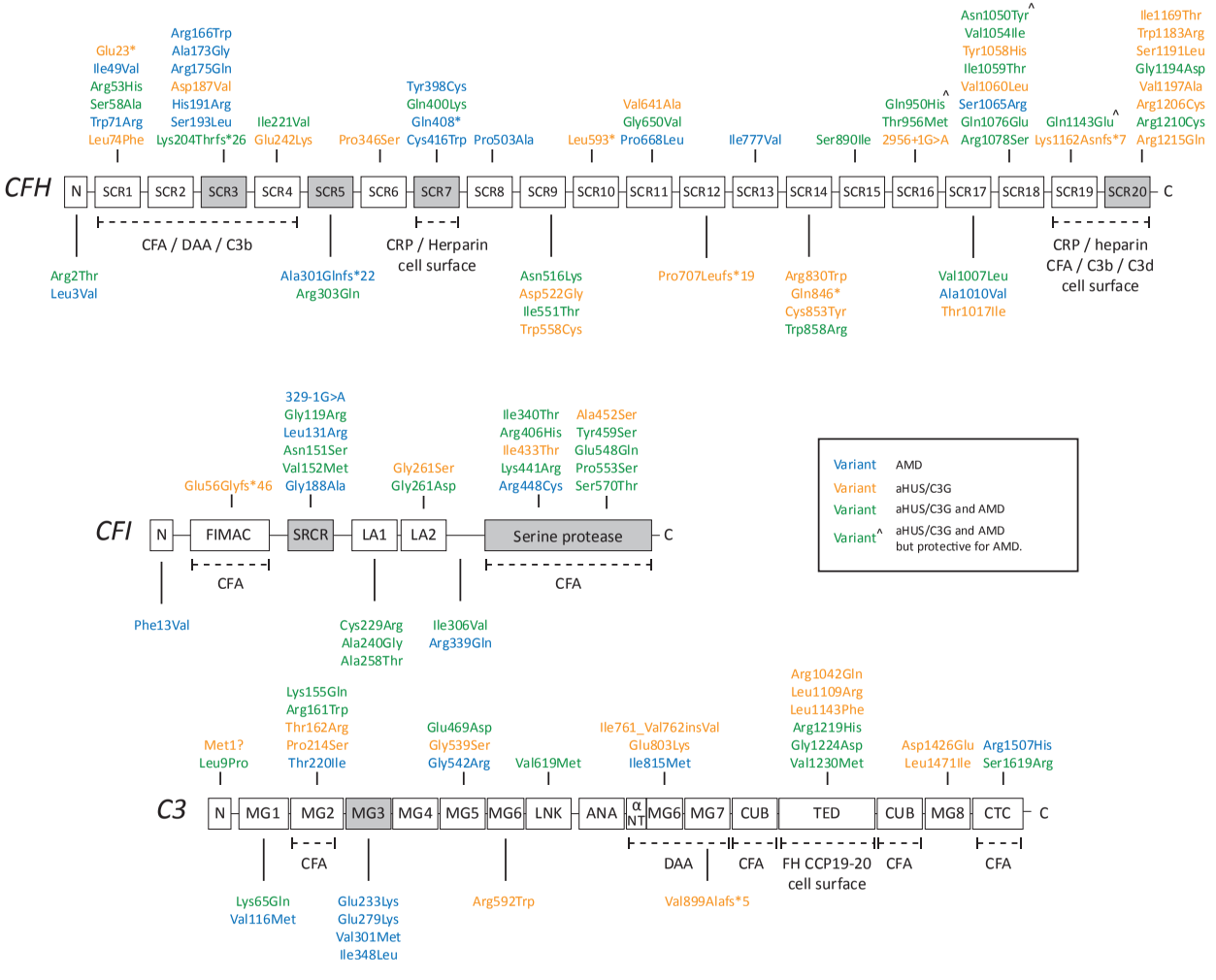
CFH
N
SCR1
SCR2
SCR3
SCR4
SCR5
SCR6
SCR7
SCR8
SCR9
SCR10
SCR11
SCR12
SCR13
SCR14
SCR15
SCR16
SCR17
SCR18
SCR19
SCR20
C
Glu23*
Ile49Val
Arg53His
Ser58Ala
Trp71Arg
Leu74Phe
Arg166Trp
Ala173Gly
Arg175Gln
Asp187Val
His191Arg
Ser193Leu
Lys204Thrfs*26
Ile221Val
Glu242Lys
Pro346Ser
Tyr398Cys
Gln400Lys
Gln408*
Cys416Trp
Pro503Ala
Leu593*
Val641Ala
Gly650Val
Pro668Leu
Ile777Val
Ser890Ile
Gln950His^
Thr956Met
2956+1G>A
Asn1050Tyr^
Val1054Ile
Tyr1058His
Ile1059Thr
Val1060Leu
Ser1065Arg
Gln1076Glu
Arg1078Ser
Gln1143Glu^
Lys1162Asnfs*7
Ile1169Thr
Trp1183Arg
Ser1191Leu
Gly1194Asp
Val1197Ala
Arg1206Cys
Arg1210Cys
Arg1215Gln
CFA / DAA / C3b
CRP / Herparin
cell surface
CRP / heparin
CFA / C3b / C3d
cell surface
Arg2Thr
Leu3Val
Ala301Glnfs*22
Arg303Gln
Asn516Lys
Asp522Gly
Ile551Thr
Trp558Cys
Pro707Leufs*19
Arg830Trp
Gln846*
Cys853Tyr
Trp858Arg
Val1007Leu
Ala1010Val
Thr1017Ile
CFI
N
FIMAC
SRCR
LA1
LA2
Serine protease
C
CFA
CFA
329-1G>A
Gly119Arg
Leu131Arg
Asn151Ser
Val152Met
Gly188Ala
Glu56Glyfs*46
Gly261Ser
Gly261Asp
Ile340Thr
Arg406His
Ile433Thr
Lys441Arg
Arg448Cys
Ala452Ser
Tyr459Ser
Glu548Gln
Pro553Ser
Ser570Thr
Phe13Val
Cys229Arg
Ala240Gly
Ala258Thr
Ile306Val
Arg339Gln
Variant AMD
Variant aHUS/C3G
Variant aHUS/C3G and AMD
Variant^ aHUS/C3G and AMD but protective for AMD.
C3
N
MG1
MG2
MG3
MG4
MG5
MG6
LNK
ANA
α NT
MG6
MG7
CUB
TED
CUB
MG8
CTC
C
CFA
DAA
CFA
FH CCP19-20
cell surface
CFA
CFA
Met1?
Leu9Pro
Lys155Gln
Arg161Trp
Thr162Arg
Pro214Ser
Thr220Ile
Glu469Asp
Gly539Ser
Gly542Arg
Val619Met
Ile761_Val762insVal
Glu803Lys
Ile815Met
Arg1042Gln
Leu1109Arg
Leu1143Phe
Arg1219His
Gly1224Asp
Val1230Met
Asp1426Glu
Leu1471Ile
Arg1507His
Ser1619Arg
Lys65Gln
Val116Met
Glu233Lys
Glu279Lys
Val301Met
Ile348Leu
Arg592Trp
Val899Alafs*5

Figure 1: Genetic variants identified in 886 aHUS/C3G patients and 697 AMD patients. Colors represent the phenotype in which the variants were identified: green (both in aHUS/C3G and AMD), orange (aHUS/C3G only), blue (AMD only) or green^ (with circumflex)(protective for AMD but found recurrently in aHUS/C3G). Protein domains that carried a significantly higher percentage of alleles in aHUS/C3G or AMD are colored gray. Protein interaction sites are reported underneath the domains with dotted lines. CFA = cofactor activity; DAA = decay-accelerating activity; CRP = C-reactive protein.

We next mapped the specific mutated amino acids found in the domains that differ significantly in allele frequency between aHUS/C3G and AMD on the available molecular structures of the C3b-FH-FI complex. These include the SCR3 and SCR20 domains of FH, the SRCR and serine protease domains of FI, and the MG3 domain of C3 in 5O32 (**Figure 2**). FH SCR5 and SCR7 we not mapped since there is no available protein structure. **Figure 3** shows a detailed view of the residues that are altered in the SCR3 domain of FH (**Figure 3a** and **b**) and the SP domain of FI (**Figure 3c** and **d**) in the 5O32 complex, together with the amino acids of interacting protein partners that are in close proximity to the altered residues.

Based on the mapping of the variants on the C3b-FH-FI protein complex, several observations can be made. Amino acids affected by aHUS/C3G variants in the SCR20 domain of FH do not interact directly with C3b (**Figure 2**). Amino acids affected by AMD variants in the SCR3 domain are located at the interface of FH with C3b and with FI (**Figure 2**). The residue Arg166 of SCR3, mutated in AMD, is located at the interface with the SP domain of FI as well as with CUB domain of C3b (**Figure 2 and 3a**). Other amino acids altered in AMD (Arg175, Ala173, His191, Asp187 and Ser193) are located at the interface with the C3b molecule. (**Figure 3b**).

Variants affecting residues in the SP domain of FI appear to have different effects on FI binding and FI activity. Residues Arg406 and Lys441, affected by genetic variants identified in aHUS/C3G and AMD, make contacts with Glu123 and Asn136 of FH, respectively (**Figure 3c**). The residues Pro553 and Glu548, affected by genetic variants identified in aHUS/C3G and AMD, are located in one of the activation loops in proximity to the FI active site (**Figure 3d**). The AMD-associated variants affecting the SRCR domain of FI, are not located at the interface with C3b or FH (**Figure 2**).

aHUS /C3G and AMD variants found in literature

To compare the results of our study to previously reported variants, we compiled a list of low frequency variants found in literature for aHUS, C3G and AMD (**Table S4**). We identified an additional 441 unique variants resulting an amino acid change or splice site in *CFH* (n=236), *CFI* (n=104), and *C3* (n=101). These variants were found in aHUS/C3G (n=212), AMD (n=189) and both phenotypes (n=40)(**Table S5**).

Table 1: Allelic distribution of *CFH, CFI* and *C3* variants identified in 886 aHUS/C3G patients and 697 AMD patients.

Gene	Domain	aHUS/C3G	AMD	p-value aHUS/C3G vs AMD
CFH	N-terminus	1 (0.4%)	1 (1.8%)	0.340
	SCR1	6 (2.5%)	3 (5.5%)	0.377
	SCR2	0 (0%)	0 (0%)	1.000
	SCR3	2 (0.8%)	8 (14.5%)	**3.20E-05**
	SCR4	3 (1.3%)	0 (0%)	1.000
	SCR5	0 (0%)	5 (9.1%)	**1.97E-04**
	SCR6	1 (0.4%)	0 (0%)	1.000
	SCR7	2 (0.8%)	5 (9.1%)	**0.003**
	SCR8	0 (0%)	1 (1.8%)	0.187
	SCR9	13 (5.4%)	1 (1.8%)	0.480
	SCR10	1 (0.4%)	0 (0%)	1.000
	SCR11	2 (0.8%)	2 (3.6%)	0.160
	SCR12	2 (0.8%)	0 (0%)	1.000
	SCR13	0 (0%)	1 (1.8%)	0.187
	SCR14	8 (3.3%)	1 (1.8%)	1.000
	SCR15	19 (7.9%)	1 (1.8%)	0.139
	SCR16	20 (8.4%)	8 (14.5%)	0.199
	SCR17	31 (13%)	2 (3.6%)	1.000
	SCR18	77 (32.2%)	14 (25.5%)	0.419
	SCR19	11 (4.6%)	0 (0%)	0.228
	SCR20	40 (16.7%)	2 (3.6%)	**0.010**
	N-terminus (SCR1-4)	11 (4.6%)	11 (20.0%)	**5.20E-04**
	C-terminus (SCR19-20)	51 (21.3%)	2 (3.6%)	**1.44E-03**
	Total CFH	239	55	
CFI	N-terminus	0 (0%)	1 (3.2%)	0.383
	FIMAC	1 (2.0%)	0 (0%)	1.000
	SRCR	7 (14.0%)	15 (48.4%)	**1.60E-03**
	LA1	3 (6.0%)	3 (9.7%)	0.670
	LA2	6 (12.0%)	2 (6.5%)	0.704
	Linker region	1 (2.0%)	1 (3.2%)	1.000
	SP	32 (64.0%)	9 (29.0%)	**2.96E-03**
	Total CFI	50	31	

Table 1 (continued)

Gene	Domain	aHUS/C3G	AMD	p-value aHUS/C3G vs AMD
C3	MG1	4 (4.4%)	3 (7.5%)	0.675
	MG2	45 (50.0%)	21 (52.5%)	0.850
	MG3	0 (0%)	5 (12.5%)	**2.30E-03**
	MG4	0 (0%)	0 (0%)	1.000
	MG5	9 (10.0%)	1 (2.5%)	0.174
	MG6a	4 (4.4%)	0 (0%)	0.311
	LNK	4 (4.4%)	1 (2.5%)	1.000
	ANA	0 (0%)	0 (0%)	1.000
	MG6b	4 (4.4%)	1 (2.5%)	1.000
	MG7	2 (2.2%)	0 (0%)	1.000
	CUB	0 (0%)	0 (0%)	1.000
	TED	8 (8.9%)	0 (0%)	0.106
	MG8	2 (2.2%)	0 (0%)	1.000
	CTC	4 (4.4%)	6 (15.0%)	0.068
	Total C3	90	40	

Percentage of variant alleles identified with significant p-values in bold.

DISCUSSION

In aHUS/C3G patients we observed a higher frequency of protein-altering alleles in the C-terminal SCR20 domain of FH and in the SP domain of FI (**Figure 1**). In AMD patients an increased frequency of protein-altering alleles was observed in the N-terminal SCR domains of FH, specifically domains SCR3, SCR5, and SCR7, in addition to the SRCR domain of FI, and the MG3 domain of C3**.**

Complement factor H

The SCR20 domain of FH, which is thought to interact with both C3b, C-reactive protein and endothelial cells, harbors significantly more aHUS/C3G associated genetic variants. Amino acids affected by genetic variants in this domain do not interact directly with C3b (Figure 2), but are likely to mediate FH attachment to the cell surface. Reduced binding to C3b and C3d was previously observed for variants p.Val1197Ala and p.Arg1210Cys in *CFH* (Table S3), which may be explained by the role of these residues in the overall structure of the FH C-terminus.

Interestingly, like SCR20, SCR19 of FH is also described to be important for interaction with the thioester-containing domain (TED) domain of C3b and C3d, and for cell surface interactions. Although the prevalence of aHUS/C3G variants in SCR19 was not significant in this study, no AMD variants were found by us in this domain. Only variant *CFH* p.Gln1143Glu, protective for AMD,[9,10] was detected (**Table S3**), indicating the importance of genetic changes in this domain for the pathogenesis of renal disease. Since phenotype-specific prevalence of genetic variants in the TED domain of C3 for renal disease was not discovered by us or described in literature (**Table S3; Table S4**), the disease-specificity of C-terminal FH variants may lay within the interactions with the endothelium.

In AMD patients, genetic variants were more prevalent in the N-terminal region of the protein. Available co-crystallization structures show that SCR3 is located at the interface of FH with C3b and with FI (**Figure 2**). The residue Arg166 of SCR3, mutated in AMD, is located at the interface with the serine protease domain of FI as well as with the CUB domain of C3b (**Figure 2 and 3a**). Other amino acids altered in AMD (Arg175, Ala173, His191, Asp187 and Ser193) are located at the interface with C3b molecule. (Figure 3b).

Our results are comparable to the variants found in literature. The variants more prevalent in aHUS/C3G are grouped at the C-terminal SCR19-20 domains of FH (52/148), while the AMD variants grouped in the first four SCR domains of *CFH* (28/74) (**Table S4**). Previously, over 60% of the aHUS-associated FH mutations were reported at the C-terminus.[21] Variants in this region interfere with heparin binding, binding of C3b and C3d, and result in reduced cell-surface interaction.[30,31] The low frequency variants reported in AMD are located at the N-terminus of FH and decrease cofactor activity for the FI-mediated cleavage of C3b.[12,22]

The C-terminus of FH is a hotspot for genetic variants in renal diseases, but not AMD, and is essential in endothelial binding of FH.[32] Interestingly, the retinal Bruch's membrane differs from the renal glomerular basement membrane in GAG binding sites.[33] It is hypothesized that not FH but Factor H Like 1 (FHL1), the short form of FH (SCR1-7), is involved in the pathogenesis of AMD. FHL-1 has the same regulatory functions as FH, but due to its size only this FH form can diffuse into the Bruch's membrane and drusen in the eye.[34,35] This may explain why the genetic alterations at the C-terminus of FH, which is not present in FHL-1, are less prevalent in AMD than in renal disease. Consistently, the SCR19–20 region of FH localizes to GAGs in the glomeruli.[33]

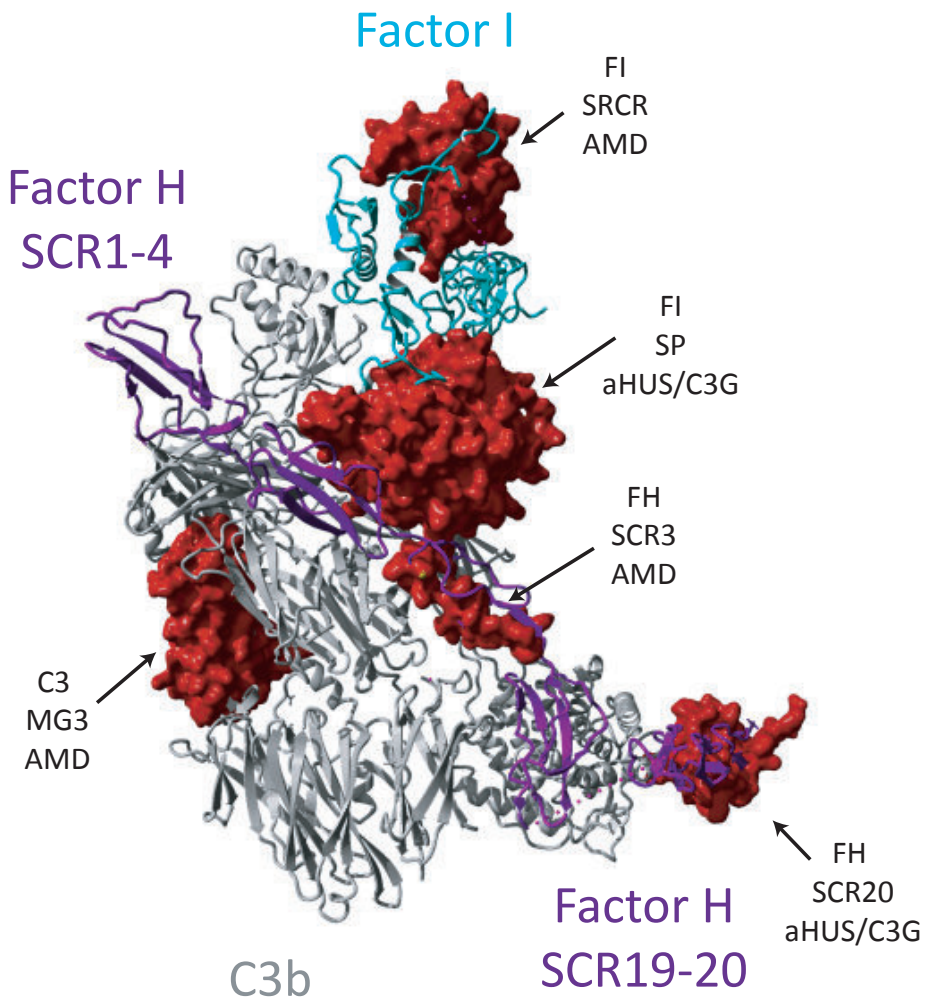

Figure 2: Location of protein domains containing a higher percentage of alleles in aHUS/C3G or AMD on the structure of the C3b-FH-FI complex. Three-dimensional structure of C3b (gray) in complex with FI (cyan) and FH construct (purple) containing FH SCR1-4 and SCR19-20. Surfaces of fragments of C3b (233-348), FI (119-188, 340-570) and FH (166-193, 1169-1215) carrying missense changes in domains, which contain a significantly higher percentage of alleles in aHUS/C3G or AMD, are shown in red. The figure is generated based on the PDB 5O32 [27], using YASARA Version 17.8.15.[29].

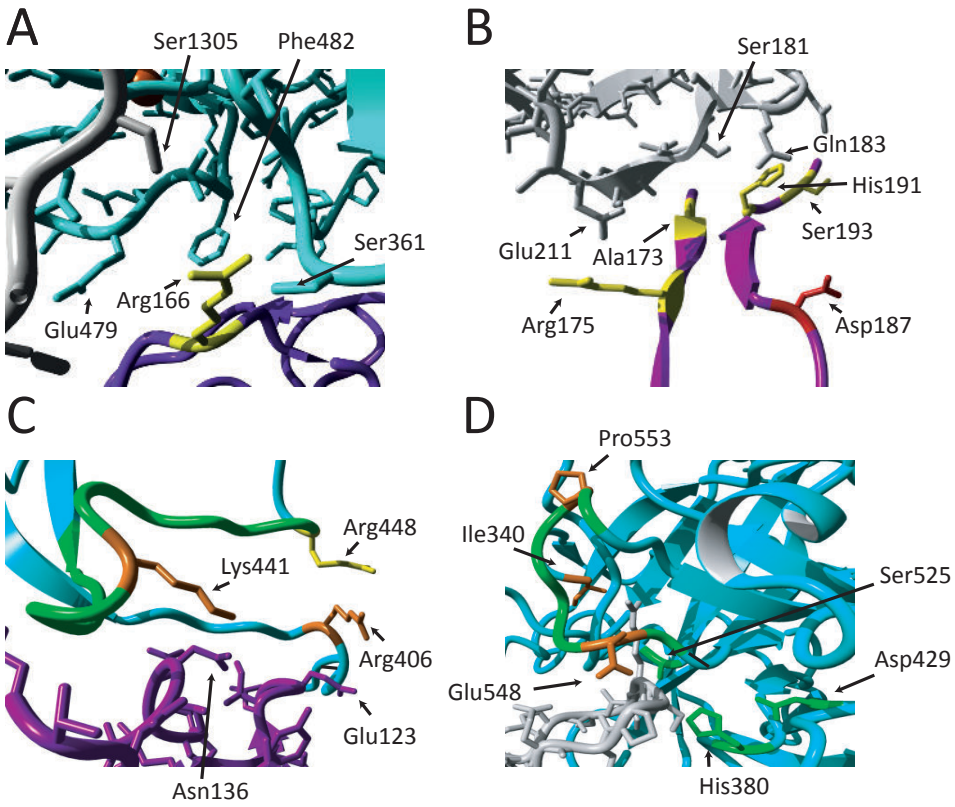

Figure 3: Localization of altered residues on the interface of C3b, FH and FI in proteins domains containing a higher percentage of alleles in aHUS/C3G or AMD. Fragments of the three dimensional structure of C3b (gray), FI (cyan) and FH construct (purple) are shown. The residues altered in the SCR3 domain of FH (A and B) and the SP domain of FI (C and D) are shown. Residues that were found mutated in AMD only (yellow), aHUS/C3G (red) or both phenotypes (orange) are indicated, as well as amino acids of interacting partners that are in close proximity of the mutated residues (A, B, C). Important structural elements of FI are indicated in green: the charged loop 435-448 (C); the activation loop 548-553 and the catalytic triad (H380, D429, S525) (D). The figure is generated based on the PDB 5O32 [27], using YASARA Version 17.8.15.[29]

Overall, C3b interactions with glomerular endothelium in the kidney may be more important than the interaction with the endothelium in the retina for maintaining the tissue homeostasis. Furthermore, our data underscore the importance of N-terminal region of FH in C3b regulation in AMD.

Three variants in *CFH* (c.2850G>T, p.Gln950His; c.3148A>T, p.Asn1050Tyr; c.3427C>G, p.Gln1143Glu) were reported as protective for AMD.[10] *CFH* p.Gln950His, residing in SCR16,

is associated with disease risk for aHUS with moderate effect on cofactor function [36]. *CFH* p.Asn1050Tyr, in SCR18, is a polymorphism reported various times in aHUS without functional analyses.[37-39] No information is available on *CFH* p.Gln1143Glu and its association with aHUS/C3G. The protective mechanisms of these variants in AMD remain to be elucidated.

Complement factor I

In this study we observed that genetic changes in the SP domain of FI are more prevalent in aHUS/C3G than in AMD patients. The SP domain, together with the FIMAC domain, contains the binding sites important for C3b and C4b degradation [40,41]. Variants found in the SP domain could interfere with proper cofactor activity . Residues Arg406 and Lys441 in the SP domain make contacts with Glu123 and Asn136 of FH, respectively (**Figure 3c**). Interestingly, a *CFI* variant affecting Arg448, found in AMD, is located in close proximity to these residues, within the charged loop (435-448) that is important in interaction with various C3b regulators (**Figure 3c).**[27] The *CFI* residues Pro553 and Glu548 are located in one of the activation loops in proximity to the FI active site (**Figure 3d**),[42] while Ile340 at the N-terminus of the light chain plays a role in stabilizing of the oxyanion hole.[27] The residue Tyr459 is a part of the hydrophobic patch, which is important in heavy chain/light chain contact of FI.[27]

Furthermore, we observed a clustering of alleles in the SRCR domain in AMD patients. None of the variants are located at the interface with C3b or FH. However, FI serum levels of AMD individuals carrying low frequency variants in the SRCR domain were reported as reduced (**Table S3; Table S4**), thus leading to overall impaired ability to degrade and inactivate C3b.

In literature, the majority of variants were identified in the FI membrane-attack complex (FIMAC) (4/24 and 10/62), SRCR (5/24 and 12/62) and serine protease (SP) (10/24 and 23/62) domains for both aHUS/C3G and AMD. Previously, a burden of genetic variants was found in the light catalytic chain, the SP domain of FI for aHUS/C3G patients,[40,41] and for AMD [1,43]. However, we observe an enrichment of genetic variants in the SRCR domain of FI. It should be noted that rare genetic variant p.Gly119Arg, which confers high risk for AMD, also resides in SRCR of FI.[14]

Thus, the FI genetic changes in AMD, which are more prevalent in the SRCR domain, are likely to affect the FI expression and thus overall activity, while FI changes more prevalent in renal disease are mostly involved in structural elements around the active site.

Complement component 3

In this study, an increased number of variants were found in the MG3 domain of C3 in AMD patients (**Table S3; Table S4**). There are no interactions known for the MG3 domain in C3b, including that with FB or complement inhibitors.[44,45] However, MG3 is located at the predicted interface between C3b and C3 in the C3bBb-C3 enzyme-substrate complex. Genetic alterations

in MG3 may thus result in changes at this interface and in more efficient C3 activation by C3 convertase.[46] The AMD variants affecting residues in the MG3 domain may thus affect the overall domain structure and the rates of C3 secretion and/or activation into C3b.

In literature, the majority of variants were identified in the TED domain for both aHUS/C3G (22/40) and AMD (14/53)(**Table S4**). For aHUS it was previously reported that the majority of variants reside in the TED, MG2 and MG5 domains of C3.[16] The majority of aHUS/C3G variants were identified in the TED (8.9%) and MG2 (50%) domains. For AMD, we did not identify any variants in the TED domain, but the majority of variants identified resided in MG2 (52.5%) as well. Interestingly, rare genetic variant p.Lys155Gln, reported to confer risk for AMD, resides in the MG2 domain.

In conclusion, our data underscore the importance of the N-terminus of FH in C3b regulation in AMD, and of the FH C-terminus in renal disease. Alterations in the SRCR domain of FI (prevalent in AMD) are likely to affect expression and thus overall FH activity, while FI changes related to renal disease are involved in structural elements around the active site. For C3, AMD alterations located the MG3 domain may alter C3 secretion and/or activation into C3b. We observed a substantial overlap in variants between aHUS/C3G and AMD, however there is a distinct clustering of variants within specific domains.

Depending on the location of the variant, the genetic variants follow a distinctive genotype–phenotype correlation. Some genetic variants are associated with aHUS, C3G and AMD but individuals carrying these risk variants only present phenotypic characteristics of one disorder.[17,47] It is likely not one specific variant that results in the manifestation of aHUS, C3G, or AMD, but a combination of rare and common disease specific risk variants and environmental factors will lead to the manifestation of either an eye or renal phenotype.

SUPPLEMENTARY INFORMATION

Table S1: Number of alleles found in this study. Total number of alleles identified in aHUS/C3G and AMD patients (heterozygous/homozygous)

	Alleles in aHUS/C3G patients (het/hom)	Alleles in AMD patients (het/hom)
CFH	239 (225/7)	55 (55/0)
CFI	50 (46/2)	31 (31/0)
C3	90 (88/1)	40 (40/0)
total	379 (359/10)	126 (126/0)

Table S2: Number of low frequency variants found in this study. Total number of genetic variants identified in aHUS/C3G and AMD patients (number of novel variants).

	aHUS/C3G specific variants (novel)	AMD specific variants (novel)	aHUS/C3G and AMD variants (novel)
CFH	24 (15)	17 (8)	23 (1)
CFI	4 (4)	6 (2)	15 (1)
C3	13 (11)	9 (8)	10 (1)
total	41 (30)	32 (18)	48 (3)

Table S3: Variants identified in this study.

Pheno type	Gene	Domain	chr	c.	p.	ExAC (%)	aHUS/ C3G total	aHUS/ C3G hm/ ht	aHUS/ C3G comment	AMD totaal	AMD hm/ht	AMD comment	Functional Analyses	Source
Both	CFH	Terminus	1	5G>C	Arg2Thr	0.002	822	0/1				Case-control analysis (OR 14.08; p=0.02)		Fritsche et al., 2016
AMD	CFH	Terminus	1	7C>G	Leu3Val	0.020				697	0/1	Case-control analysis (OR 1.06; p=0.89)		Fritsche et al., 2016
aHUS/C3G	CFH	SCR1	1	67G>T	Glu23*	NA	822	0/1						Unreported
AMD	CFH	SCR1	1	145A>G	Ile49Val	0.002				697	0/1			Unreported
Both	CFH	SCR1	1	158G>A	Arg53His	NA	822	0/1	Reported in aHUS			Case-control analysis (OR 13.39; p=0.01)	Minor decreased affinity to bind C3b. Independent of the C3b affinity, the variant strongly affected co-factor activity of FI. In addition, the variant disrupted decay accelerating activity and was shown to correlate to low C3 levels.	Fritsche et al., 2016; Janssen van Doorn et al., 2013; Pechtl et al., 2011; Saunders et al 2007
Both	CFH	SCR1	1	172T>G	Ser58Ala	0.010	822	0/1	Reported in aHUS	697	0/1	Case-control analysis (OR 2.82; p=0.007)		Fritsche et al., 2016; Rodriguez de Cordoba et al., 2014
AMD	CFH	SCR1	1	211T>A	Trp71Arg	NA				697	0/1			Unreported
aHUS/C3G	CFH	SCR1	1	220C>T	Leu74Phe	0.001	822	0/3						Unreported
AMD	CFH	SCR3	1	496C>T	Arg166Trp	NA				697	0/1	Case-control analysis		Triebwasser et al., 2015
AMD	CFH	SCR3	1	518C>G	Ala173Gly	NA				697	0/1	Case-control analysis		Duvvari et al., 2015

AMD	CFH	SCR3	1	524G>A	Arg175Gln	NA				697	0/2	Segregation analysis	No reported effect on FH levels. Reduced C3b degradation ability.	Geerlings et al., 2017
aHUS/C3G	CFH	SCR3	1	560A>T	Asp187Val	NA	822	0/1						Unreported
AMD	CFH	SCR3	1	572A>G	His191Arg	NA				697	0/1			Unreported
AMD	CFH	SCR3	1	578C>T	Ser193Leu	NA				697	0/3	Segregation analysis	No reported effect on FH levels. Reduced C3b degradation ability.	Geerlings et al., 2017
Both	CFH	SCR3	1	607_610 dupCCAA	Lys204Thrfs*26	NA	822	0/1				Segregation analysis		Van de Ven et al., 2012
Both	CFH	SCR4	1	661A>G	Ile221Val	0.003	822	0/2				Case-control analysis (OR 11.80; p=0.03)		Fritsche et al., 2016
aHUS/C3G	CFH	SCR4	1	724G>A	Glu242Lys	NA	822	0/1						Unreported
AMD	CFH	SCR5	1	901del	Ala301Glnfs*22	NA				697	0/2	Case-control analysis		Duvvari et al., 2015
Both	CFH	SCR5	1	908G>A	Arg303Gln	0.002			Reported in HELLP syndrome	697	0/3	Case-control analysis (OR 9.47; p=0.08)	Normal plasma levels for FH, FI and C3	Fakhouri et al., 2008; Fritsche et al., 2016
aHUS/C3G	CFH	SCR6	1	1036C>T	Pro346Ser	NA	822	0/1						Unreported
AMD	CFH	SCR7	1	1193A>G	Tyr398Cys	0.001				697	0/1			Unreported
Both	CFH	SCR7	1	1198C>A	Gln400Lys	0.008	822	0/2		697	0/1	Case-control analysis (OR 0.91; p=0.87)	Lower FH levels, but no effect on plasma concentrations of C3 and FB	Dragon-Durey et al., 2004; Fritsche et al., 2016; Duvvari et al., 2015; Triebwasser et al., 2015; Geerdink et al 2012
AMD	CFH	SCR7	1	1222C>T	Gln408*	NA				697	0/2	Case-control analysis		Boon et al., 2008
AMD	CFH	SCR7	1	1248C>G	Cys416Trp	NA				697	0/1			Unreported

Pheno type	Gene	Domain	chr	c.	p.	ExAC (%)	aHUS/ C3G total	aHUS/ C3G hm/ ht	aHUS/ C3G comment	AMD totaal	AMD hm/ht	AMD comment	Functional Analyses	Source
AMD	CFH	SCR8	1	1507C>G	Pro503Ala	NA				697	0/1	Segregation analysis		Hoffman et al., 2014; Triebwasser et al., 2015; Zhan et al., 2013
Both	CFH	SCR9	1	1548T>A	Asn516Lys	0.040	822	0/1				Case-control analysis (OR 2.05; p=0.07)		Fritsche et al., 2016; Le Quintrec 2008; Bresin et al 2013; Zhang et al 2012
aHUS/C3G	CFH	SCR9	1	1565A>G	Asp522Gly	NA	822	0/1						Unreported
Both	CFH	SCR9	1	1652T>C	Ile551Thr	0.500	822	0/10		697	0/1	Case-control analysis (OR 0.41; p=0.37)		Fritsche et al., 2016; Triebwasser et al., 2015; Zhan et al., 2013
aHUS/C3G	CFH	SCR9	1	1674G>C	Trp558Cys	NA	822	0/1						Unreported
aHUS/C3G	CFH	SCR10	1	1778T>A	Leu593*	NA	822	0/1						Unreported
aHUS/C3G	CFH	SCR11	1	1922T>C	Val641Ala	0.003	822	0/2						Unreported
Both	CFH	SCR11	1	1949G>T	Gly650Val	0.020			Reported in glomerulone-phritis	697	0/1	Case-control analysis (OR 1.52; p=0.33)		Fritsche et al., 2016; Triebwasser et al., 2015; Zhan et al., 2013; Servais et al 2007
AMD	CFH	SCR11	1	2003C>T	Pro668Leu	0.002				697	0/1			Unreported
aHUS/C3G	CFH	SCR12	1	2120delC	Pro707Leufs*19	NA	822	0/2						Unreported
AMD	CFH	SCR13	1	2329A>G	Ile777Val	0.002				697	0/1			Unreported
aHUS/C3G	CFH	SCR14	1	2488C>T	Arg830Trp	0.008	822	0/1						Unreported
aHUS/C3G	CFH	SCR14	1	2536C>T	Gln846*	NA	822	0/3						Unreported
aHUS/C3G	CFH	SCR14	1	2558G>A	Cys853Tyr	NA	822	0/3						Unreported
Both	CFH	SCR14	1	2572T>A	Trp858Arg	NA	822	0/1		697	0/1			Unreported

Both	CFH	SCR15	1	2669G>T	Ser890Ile	1.990	822	2/15	Reported in aHUS	697	0/1	Case-control analysis (OR 1.03; p=0.86)	Did not result is differences in FH co-activity with FI. The C3b binding was not affected and FH concentration in plasma were normal. In addition a hemolytic assay showed that the capacity to regulate the alternative pathway on cellular surfaces was normal.	Tortajada et al., 2012; Caprioli et al., 2006; Noris et al 2005
Both	CFH	SCR16	1	2850G>T	Gln950His	0.360	822	0/15	Reported in aHUS	697	0/8	Protective for AMD (OR 0.72; p=0.003)	Demonstrated reduced erythrocyte binding and, consequently, increased lysis after serum addition to sheep erythrocytes. Patient plasma levels of FH were not different compared to controls, but transient expression levels of mutant lagged behind that of the wild type. No impaired cofactor binding for FI was observed and normal complementary inhibitory functions were observed.	Mohlin et al., 2015; Szarvas et al., 2016; Fritsche et al., 2016; Caprioli et al., 2003

Pheno type	Gene	Domain	chr	c.	p.	ExAC (%)	aHUS/ C3G total	aHUS/ C3G hm/ ht	aHUS/ C3G comment	AMD totaal	AMD hm/ht	AMD comment	Functional Analyses	Source
Both	CFH	SCR16	1	2867C>T	Thr956Met	0.120	822	0/1	Reported in aHUS			Case-control analysis (OR 1.04; p=0.83)	No effect on C3 or FH levels in plasma. The lysis of erythrocytes was not increased and no effect on protein expression was shown.	Perez-Caballero et al., 2001; Szarvas et al., 2016
aHUS/C3G	CFH	SCR16	1	2956+1G>A	splicing	NA	822	0/4	Reported in aHUS					Westra et al., 2010
Both	CFH	SCR17	1	3019G>T	Val1007Leu	2.640	822	4/21	Reported in aHUS	697	0/1	Case-control analysis (OR 0.99; p=0.96)	No differences in FH co-activity nor C3b binding; normal hemolytic assay (capacity to regulate the alternative pathway on cellular surfaces); normal FH in plasma	Tortajada et al., 2012; Fritsche et al., 2016; Triebwasser et al., 2015; Zhan et al., 2013
AMD	CFH	SCR17	1	3029C>T	Ala1010Val	NA				697	0/1	Case-control analysis		Triebwasser et al., 2015
aHUS/C3G	CFH	SCR17	1	3050C>T	Thr1017Ile	0.120	822	0/2						Unreported
Both	CFH	SCR18	1	3148A>T	Asn1050Tyr	1.510	822	1/20	Reported in aHUS	697	0/11	Protective for AMD (OR 0.36; p<0.001)		Stahl et al., 2008; Fritsche et al., 2016; Boon et al., 2008; Neumann et al., 2003
Both	CFH	SCR18	1	3160G>A	Val1054Ile	0.020			Reported in aHUS	697	0/1			Rodriguez de Cordoba et al, 2014
aHUS/C3G	CFH	SCR18	1	3172T>C	Tyr1058His	0.080	822	0/17	Reported in aHUS					Matsumoto et al 2014

Both	CFH	SCR18	1	3176T>C	Ile1059Thr	0.680	822	0/10	Reported in aHUS			Case-control analysis (OR 0.32; p=0.22)	Fritsche et al., 2016; Triebwasser et al., 2015; Zhan et al., 2013; Matar et al 2014
aHUS/C3G	CFH	SCR18	1	3178G>C	Val1060Leu	0.080	822	0/15	Reported in aHUS				Matsumoto et al 2014
AMD	CFH	SCR18	1	3193A>C	Ser1065Arg	NA				697	0/1		Unreported
Both	CFH	SCR18	1	3226C>G	Gln1076Glu	0.040	822	0/12				Case-control analysis	Triebwasser et al., 2015
Both	CFH	SCR18	1	3234G>T	Arg1078Ser	0.006	822	0/1		697	0/1	Segregation analysis	Boon et al., 2008
Both	CFH	SCR19	1	3427C>G	Gln1143Glu	0.970	822	0/9				Protective for AMD (OR 0.21; p=0.06)	Fritsche et al., 2016; Triebwasser et al., 2015; Zhan et al., 2013
aHUS/C3G	CFH	SCR19	1	3486delA	Lys1162Asnfs*7	NA	822	0/2					Richards et al., 2001
aHUS/C3G	CFH	SCR20	1	3506T>C	Ile1169Thr	NA	822	0/2					Unreported
aHUS/C3G	CFH	SCR20	1	3547T>C	Trp1183Arg	NA	822	0/2	Reported in aHUS				No effect on the cofactor activity, impaired interaction with surface-bound C3b	Sánchez-Corral et al., 2002; Neumann et al 2003
aHUS/C3G	CFH	SCR20	1	3572C>T	Ser1191Leu	NA	822	0/18	Reported in aHUS				High LD with Val1197Ala	Heinen et al., 2006; Sanchez-Corral et al., 2002; Ying et al 1999
Both	CFH	SCR20	1	3581G>A	Gly1194Asp	0.003			Reported in aHUS	697	0/1	Case-control analysis (OR 7.41; p=0.12)	Slightly increased complement regulatory function of mutant FH on cell surfaces (sheep erythrocyte lysis); normal C3 FH and FI levels in serum	Bresin et al., 2013; Johnson et al., 2010; Caprioli et al., 2003; Perkins et al 2002

Pheno type	Gene	Domain	chr	c.	p.	ExAC (%)	aHUS/ C3G total	aHUS/ C3G hm/ ht	aHUS/ C3G comment	AMD totaal	AMD hm/ht	AMD comment	Functional Analyses	Source
aHUS/C3G	CFH	SCR20	1	3590T>C	Val1197Ala	NA	822	0/12	Reported in aHUS				Normal lysis of sheep erythrocytes, low FH and C3 levels, and shows low binding to surface bound C3b.	Heinen et al., 2006; Sanchez-Corral et al., 2002; Richards et al 2001
aHUS/C3G	CFH	SCR20	1	3616C>T	Arg1206Cys	NA	822	0/3						Westra et al 2010
Both	CFH	SCR20	1	3628C>T	Arg1210Cys	0.020	822	0/2	Reported in aHUS	697	0/1	Case-control analysis (OR 20.28; p<0.001)	This variant results in a covalent binding to human serum albumin which hampers all FH functional domains. It also shows reduced binding to heparin and endothelial cells and binding to C3b and C3d is also decreased. No effects on cofactor activity for FI was reported and no effect on erythrocyte lysis was shown.	Ferreira et al., 2009; Jozsi et al., 2006; Manuelian et al., 2003; Recalde et al., 2016; Sanchez-Corral et al., 2002; Zhan et al., 2013; Fritsche et al., 2016; Triebwasser et al., 2015; Miyake et al., 2015; Raychaudhuri et al., 2011
aHUS/C3G	CFH	SCR20	1	3644G>A	Arg1215Gln	NA	822	0/1	Reported in aHUS				Strong lysis, decreased affinity to heparin, normal binding to C3b/C3d and to endothelial cells	Lehtinen et al. 2009; Caprioli et al. 2001; Szarvas et al., 2016

Pheno type	Gene	Domain	chr	c.	p.	ExAC (%)	aHUS/C3G total	aHUS/ C3G hm/ ht	aHUS/C3G comment	AMD totaal	AMD hm/ht	AMD comment	Functional Analyses	Source
AMD	CFI	Terminus	4	37T>G	Phe13Val	0.001				697	0/1			Unreported
aHUS/C3G	CFI	FIMAC	4	167delA	Glu56Glyfs*46	NA	793	0/1						Unreported
AMD	CFI	SRCR	4	329-1G>A	Splicesite	NA				697	0/1			Unreported
Both	CFI	SRCR	4	355G>A	Gly119Arg	0.050	793	0/3	Reported in aHUS	697	0/11	Case-control analysis (OR 3.64; p<0.001)	Reduced FI levels in human serum and in transient in vitro expression studies. Results in a lower ability to degrade C3b due to impaired expression and secretion of the mutant protein.	Fritsche et al., 2016; van de Ven et al., 2013; Geerlings et al., 2016; Kavanagh et al., 2015; Alexander et al., 2014; Tan et al., 2017; Maga et al., 2010
AMD	CFI	SRCR	4	392T>G	Leu131Arg	NA				697	0/2	Segregation analysis	The variant resulted in a lower ability to degrade C3b which could be due to impaired expression and secretion of the mutant protein.	Geerlings et al., 2016
Both	CFI	SRCR	4	452A>G	Asn151Ser	0.001	793	0/2	Reported in aHUS			Case-control analysis (OR 0.74; p=0.53)	Reported as quantitative deficiency with reduced FI levels in supernatant and lysate of transfected cells. Normal FI in vivo assay of retinal vascularization in zebrafish.	Bienaime et al, 2010; Fritsche et al., 2016; Tan et al., 2017; Bresin et al., 2013; Nilsson et al., 2010
Both	CFI	SRCR	4	454G>A	Val152Met	0.002	793	0/2	Reported in aHUS			Case-control analysis (OR 7.57; p<0.001)	Normal FI in in vivo assay of retinal vascularization in zebrafish but low FI serum in two AMD patients.	Kavanagh et al., 2015; Tan et al., 2017; Fritsche et al., 2016; Westra et al., 2010; Seddon et al., 2013

Pheno type	Gene	Domain	chr	c.	p.	ExAC (%)	aHUS/C3G total	aHUS/ C3G hm/ ht	aHUS/C3G comment	AMD totaal	AMD hm/ht	AMD comment	Functional Analyses	Source
AMD	CFI	SRCR	4	563G>C	Gly188Ala	NA				697	0/1		Resulted in lower FI levels in human serum as well as in transient in vitro expression studies. The variant resulted in impaired degradation of C3b.	van de Ven et al., 2013; Alexander et al., 2014
Both	CFI	LA1	4	685T>C	Cys229Arg	NA	793	0/1				Case-control analysis		Kavanagh et al., 2015
Both	CFI	LA1	4	719C>G	Ala240Gly	0.030			Reported in aHUS	697	0/1	Case-control analysis	This variant resulted in lower or normal FI levels in human serum/ plasma. The degradation of fluid phase C4b and C3b was normal, although the ability to cleave surface-bound C3b was impaired.	Caprioli et al., 2006; Kavanagh et al., 2015; Nilsson et al., 2010; Seddon et al., 2013
Both	CFI	LA1	4	772G>A	Ala258Thr	0.010	793	0/2	Reported in aHUS	697	0/2	Case-control analysis (OR 3.88; p<0.001)	Interruption of the donor splice site for the mRNA and skipping of exon 5. Reduced FI levels in human serum.	Fritsche et al., 2016; Kavanagh et al., 2015; Ponce-Castro et al., 2008; Vyse et al., 1996; Sullivan et al., 2010
aHUS/C3G	CFI	LA2	4	781G>A	Gly261Ser	0.001	793	0/6		697	0/0			Unreported

Both	CFI	LA2	4	782G>A	Gly261Asp	0.130			Reported in aHUS	697	0/2	Case-control analysis (OR 0.86; p=0.34)	Normal FI serum levels and normal FI in in vivo assay of retinal vascularization in zebrafish. Slightly different migration pattern; normal degradation of C3b and C4b. Increased C3d levels in one carrier but no difference was seen in expression, splicing or glycosylation.	Nilsson et al., 2007; Kavanagh et al., 2015; Fritsche et al., 2016; Tan et al., 2017; Servais et al., 2007; Bresin et al., 2013
Both	CFI	linker region	4	916A>G	Ile306Val	0.050	793	0/1				Case-control analysis (OR 2.417; p=0.59)		Fritsche et al., 2016
AMD	CFI	linker region	4	1016G>A	Arg339Gln	NA			Reported in aHUS	697	0/1	Case-control analysis (OR 11.834; p=0.03)	Reduced C3, FH, and FB levels, but normal FI levels in serum	Fritsche et al., 2016; Kavanagh et al., 2015; Zhan et al., 2013; Szarvas et al., 2016
Both	CFI	SP	4	1019T>C	Ile340Thr	0.004	793	0/1	Reported in aHUS			Case-control analysis (OR 1.68; p=0.33)	Normal FI and C3 levels in serum	Fritsche et al., 2016; Kavanagh et al., 2015; Bresin et al., 2013; Geelen et al., 2007; Geerdink et al., 2012; Haerynck et al., 2013
Both	CFI	SP	4	1217G>A	Arg406His	1.690	793	1/6		697	0/2	Case-control analysis (OR 1.25; p=0.47)	No effect on FI levels in human serum and normal FI in vivo assay of retinal vascularization in zebrafish.	Kavanagh et al., 2015; Fritsche et al., 2016; Tan et al., 2017
aHUS/C3G	CFI	SP	4	1298T>C	Ile433Thr	NA	793	0/2						Unreported

Pheno type	Gene	Domain	chr	c.	p.	ExAC (%)	aHUS/C3G total	aHUS/ C3G hm/ ht	aHUS/C3G comment	AMD totaal	AMD hm/ht	AMD comment	Functional Analyses	Source
Both	CFI	SP	4	1322A>G	Lys441Arg	0.340	793	0/3	Reported in aHUS	697	0/1	Case-control analysis (OR 1.15; p=0.24)	Normal FI serum levels and normal FI in vivo assay of retinal vascularization in zebrafish.	Cayci et al., 2012; Kavanagh et al., 2015; Fritsche et al., 2016; Tan et al., 2017; Abouelhoda et al., 2016; Seddon et al., 2013
AMD	CFI	SP	4	1342C>T	Arg448Cys	0.002				697	0/1		Hypoactive complement factor I in in vivo assay of retinal vascularization in zebrafish (enlarged hyaloid vessels)	Tan et al., 2017
aHUS/C3G	CFI	SP	4	1354G>T	Ala452Ser	0.001	793	0/1						Unreported
Both	CFI	SP	4	1376A>C	Tyr459Ser	NA			Reported in aHUS	697	0/1	Case-control analysis (OR 2.381; p=0.27)		Bienaime et al, 2009; Kavanagh et al., 2015; Fritsche et al., 2016; Bresin et al., 2013; Seddon et al., 2013
Both	CFI	SP	4	1642G>C	Glu548Gln	0.070	793	1/10						Zhan et al., 2013
Both	CFI	SP	4	1657C>T	Pro553Ser	0.130	793	0/3	Reported in aHUS	697	0/3	Case-control analysis	This variant had no effect on FI levels in human serum and slightly lower ability to degrade C3b. Normal FI in vivo assay of retinal vascularization in zebrafish.	Geerlings et al., 2016; Kavanagh et al., 2015; Tan et al., 2017; Fang et al., 2008; Bresin et al., 2013
Both	CFI	SP	4	1709G>C	Ser570Thr	0.004	793	0/2		697	0/1			Unreported

Pheno type	Gene	Domain	chr	c.	p.	ExAC (%)	aHUS/ C3G total	aHUS/ C3G hm/ ht	aHUS/C3G comment	AMD totaal	AMD hm/ht	AMD comment	Functional Analyses	Source
aHUS/C3G	C3	Terminus	19	1A>C	Startloss(Met1?)	NA	589	0/1						Unreported
Both	C3	Terminus	19	26T>C	Leu9Pro	0.055	589	0/3		697	0/2			Unreported
Both	C3	MG1	19	193A>C	Lys65Gln	0.006	589	0/4	Reported in aHUS	697	0/2		This variants weakened the interaction of C3b and FH and showed reduced MCP binding affinity	Volokhina et al., 2012; Duvvari et al., 2014; Schramm et al., 2015
AMD	C3	MG1	19	346G>A	Val116Met	NA				697	0/1			Unreported
Both	C3	MG2	19	463A>C	Lys155Gln	0.336	589	0/9	Reported in dense deposit disease	697	0/18	Case-control analysis (OR 2.87; p<0.001)	This variant resulted in significantly reduced cleavage of C3b in fluid phase cofactor assays as well as reduced binding to FH. MCP cofactor activity was not changed.	Fritsche et al., 2016; Helgason et al., 2013; Seddon et al., 2013; Zhan et al., 2013
Both	C3	MG2	19	481C>T	Arg161Trp	NA	589	0/33	Reported in aHUS	697	0/2	Segregation analysis	Reduced binding activity of C3b to FH in one study and no effect on binding and cleavage of C3 in other studies. MCP binding was reduced, FB binding was increased. This variant is discussed to be a gain-of-function variant of the convertase complex and C3a, C5a, C5b-9 formation was shown to be increased.	Geerlings et al., 2016; Martinez-Barricarte et al., 2015; Roumenina et al., 2012; Schramm et al., 2015; Volokhina et al., 2012; Fakhouri et al., 2010; Bresin et al., 2013

Pheno type	Gene	Domain	chr	c.	p.	ExAC (%)	aHUS/ C3G total	aHUS/ C3G hm/ ht	aHUS/C3G comment	AMD totaal	AMD hm/ht	AMD comment	Functional Analyses	Source
aHUS/C3G	C3	MG2	19	485C>G	Thr162Arg	NA	589	0/2	Reported in aHUS					Noris et al., 2010; Schramm et al., 2015
aHUS/C3G	C3	MG2	19	640C>T	Pro214Ser	NA	589	0/1						Unreported
AMD	C3	MG2	19	659C>T	Thr220Ile	NA				697	0/1			Unreported
AMD	C3	MG3	19	697G>A	Glu233Lys	0.002				697	0/1	Case-control analysis (OR 0.76; p=0.75)		Fritsche et al., 2016
AMD	C3	MG3	19	835G>A	Glu279Lys	0.004				697	0/1			Unreported
AMD	C3	MG3	19	901G>A	Val301Met	NA				697	0/2			Unreported
AMD	C3	MG3	19	1042A>C	Ile348Leu	0.005				697	0/1			Unreported
Both	C3	MG5	19	1407G>C	Glu469Asp	0.394	589	1/6	Reported in aHUS			Case-control analysis (OR 0.90; p=0.87)		Schramm et al., 2015
aHUS/C3G	C3	MG5	19	1615G>A	Gly539Ser	NA	589	0/1						Unreported
AMD	C3	MG5	19	1624G>C	Gly542Arg	NA				697	0/1			Unreported
aHUS/C3G	C3	MG6	19	1774C>T	Arg592Trp	0.001	589	0/4	Reported in aHUS				Decreased cofactor activity, reduced binding with MCP but normal binding to FH and FB.	Frémeaux-Bacchi et al., 2008; Noris et al, 2010; Al-Akash SI., 2011; Martínez-Barricarte et al., 2015; Schramm et al., 2015
Both	C3	LNK	19	1855G>A	Val619Met	0.029	589	0/4		697	0/1	Case-control analysis (OR 2.66; p<0.001)		Fritsche et al., 2016
aHUS/C3G	C3	MG6	19	2281_2283dupATC	Ile761_ Val762insVal		589	0/1						Unreported
aHUS/C3G	C3	MG6	19	2407G>A	Glu803Lys	NA	589	0/3						Unreported
AMD	C3	MG6	19	2445C>G	Ile815Met	NA				697	0/1			Unreported
aHUS/C3G	C3	MG7	19	2696delT	Val899Alafs*5		589	0/2						Unreported

aHUS/C3G	C3	TED	19	3125G>A	Arg1042Gln	NA	589	0/1					Unreported
aHUS/C3G	C3	TED	19	3326T>G	Leu1109Arg	NA	589	0/1					Unreported
aHUS/C3G	C3	TED	19	3427C>T	Leu1143Phe	NA	589	0/1					Unreported
Both	C3	TED	19	3656G>A	Arg1219His	0.004	589	0/2				Case-control analysis (OR 1.05; p=0.95)	Fritsche et al., 2016
Both	C3	TED	19	3671G>A	Gly1224Asp	0.184	589	0/2				Case-control analysis (OR 0.47; p=0.34)	Fritsche et al., 2016
Both	C3	TED	19	3688G>A	Val1230Met	0.002	589	0/1				Case-control analysis (OR 3.49; p=0.44)	Fritsche et al., 2016
aHUS/C3G	C3	MG8	19	4278C>G	Asp1426Glu	NA	589	0/1					Unreported
aHUS/C3G	C3	MG8	19	4411C>A	Leu1471Ile	NA	589	0/1					Unreported
AMD	C3	CTC	19	4520G>A	Arg1507His	0.005				697	0/1		Unreported
Both	C3	CTC	19	4855A>C	Ser1619Arg	0.110	589	0/4	Reported in aHUS	697	0/5	Case-control analysis (OR 0.90; p=0.71)	Fritsche et al., 2016; Duvvari et al., 2014; Feng et al., 2013; Bu et al., 2014

Table S4: Variants identified in literature.

Phenotype	Gene	Domain	c.	p.	ExAC (%)	aHUS/C3G comment	AMD comment	Functional Analyses	Source
AMD	CFH	SCR1	70C>G	Leu24Val	NA		Case-control analysis (OR 3.162; p=0.48)		Triebwasser et al., 2015; Zhan et al., 2013
AMD	CFH	SCR1	76C>T	Pro26Ser	NA				Triebwasser et al., 2015
aHUS/C3G	CFH	SCR1	79_82delAGAA	Arg27Glufs*6	NA				Warwicker et al., 1998
aHUS/C3G	CFH	SCR1	Not described	Ile32*	NA				Kavanagh et al, 2013
AMD	CFH	SCR1	136A>G	Thr46Ala	0.001				Triebwasser et al., 2015
Both	CFH	SCR1	157C>T	Arg53Cys	0.002	Reported in aHUS	Case-control analysis (OR 22.54; p=0.001); segregation analysis	Variant did not affect levels of FH in serum but showed slightly reduced binding affinity to C3b compared to wild type. Marked loss of decay accelerating activity. Trend towards lower cofactor activity for FI.	Fritsche et al., 2016; Triebwasser et al., 2015; Yu et al., 2014; Zhan et al., 2013; Szarvas et al, 2016; Fakhouri et al 2010
AMD	CFH	SCR1	164G>A	Gly55Glu	NA				Zhan et al., 2013
AMD	CFH	SCR1	206G>A	Gly69Glu	NA				Raychaudhuri et al., 2011
aHUS/C3G	CFH	SCR1	232A>G	Arg78Gly	NA	Reported in aHUS			Caprioli et al., 2003; Pechtl et al., 2011
aHUS/C3G	CFH	SCR1	242A>C	Gln81Pro	NA	Reported in aHUS			Bresin et al, 2013; Fakhouri et al 2010
AMD	CFH	SCR1	244+2T>C	Splicesite	NA			Normal expression of FH and high C3 in plasma of one carrier	Triebwasser et al., 2015
aHUS/C3G	CFH	SCR1	245A>G	Lys82Arg	0.002	Reported in aHUS			Burel et al., 2017
aHUS/C3G	CFH	SCR1	249G>T	Arg83Ser	NA	Reported in glomerulonephritis		Reduced C3b binding, cofactor activity, and decay accelerating activity	Wong et al., 2014
aHUS/C3G	CFH	SCR1	262C>A	Pro88Thr	NA	Reported in glomerulonephritis			Alfandary et al., 2015

AMD	CFH	SCR2	269A>G	Asp90Gly	NA		Segregation analysis	No effect on FH levels in serum levels. No effect on C3b binding affinity and decay accelerating activity. Significantly reduced FI cofactor activity	yu et al., 2014
AMD	CFH	SCR2	272C>G	Thr91Ser	0.002				Triebwasser et al., 2015
aHUS/C3G	CFH	SCR2	332T>A	Val111Glu	0.010	Reported in aHUS			Bresin et al, 2013
aHUS/C3G	CFH	SCR2	351delG	Tyr118Ilefs*4	NA	Reported in aHUS			Saunders et al., 2007
AMD	CFH	SCR2	368A>G	Glu123Gly	NA		Case-control analysis (OR 0.364; p=0.53)		Zhan et al., 2013
aHUS/C3G	CFH	SCR2	371_397del	Ile124_ Gly133delinsArg	NA				Dragon-Durey 2004
Both	CFH	SCR2	380G>A	Arg127His	0.002		Case-control analysis (OR 5.507; p=0.23)	Reduced FH serum levels in heterozygous and homozygous carriers and no secretion of the protein.	Fritsche et al., 2016; Triebwasser et al., 2015; Wagner2016; Zhan et al., 2013; Falcao et al., 2008; Albuquerque et al., 2012
aHUS/C3G	CFH	SCR2	380G>T	Arg127Leu	NA	Reported in glomerulonephritis			Dragon-Durey 2004
AMD	CFH	SCR2	386G>A	Cys129Tyr	NA				Triebwasser et al., 2015
Both	CFH	SCR2	388G>A	Asp130Asn	0.009	Reported in glomerulonephritis			Triebwasser et al., 2015; Servais et al 2012
aHUS/C3G	CFH	SCR2	400T>C	Trp134Arg	NA	Reported in aHUS			Bresin et al, 2013; Kim et al 2011
AMD	CFH	SCR2	415C>G	Pro139Ala	NA				Hughes et al., 2016
aHUS/C3G	CFH	SCR2	415C>T	Pro139Ser	NA	Reported in glomerulonephritis			Schejbel et al 2011
aHUS/C3G	CFH	SCR2	427G>A	Val143Ile	NA	Reported in dense deposit disease			Servais et al 2012
AMD	CFH	SCR3	428-2A>G	Splicesite	NA				Duvvari2015
AMD	CFH	SCR3	476G>A	Ser159Asn	0.004		Case-control analysis (OR 4.571; p=0.09)		Fritsche et al., 2016; Triebwasser et al., 2015
Both	CFH	SCR3	481G>T	Ala161Ser	0.004	Reported in aHUS			Duvvari2015; Triebwasser et al., 2015; Sellier-Leclerc et al 2007; Servais et al 2012

Phenotype	Gene	Domain	c.	p.	ExAC (%)	aHUS/C3G comment	AMD comment	Functional Analyses	Source
aHUS/C3G	CFH	SCR3	484A>G	Met162Val	0.002	Reported in aHUS			Fremeaux-Bacchi et al 2013
AMD	CFH	SCR3	497G>A	Arg166Gln	0.030				Triebwasser et al., 2015
aHUS/C3G	CFH	SCR1	497G>T	Arg166Leu	NA	Reported in aHUS			Chaudhary et al, 2014
AMD	CFH	SCR3	499G>C	Glu167Gln	NA				Triebwasser et al., 2015
AMD	CFH	SCR3	506A>G	His169Arg	0.002				Triebwasser et al., 2015
AMD	CFH	SCR3	524G>C	Arg175Pro	NA		Segregation analysis	Reduced FH serum levels and no secretion of the recombinant protein.	Triebwasser et al., 2015; Wagner2016; Zhan et al., 2013
AMD	CFH	SCR3	550delA	Ile184Leufs*33	NA		Segregation analysis		VandeVen et al., 2012
aHUS/C3G	CFH	SCR3	565G>T	Glu189*	NA			This mutation is described as causing FH deficiency and not HUS. The mutant allele generates a truncated product causing the deficiency of both FH and FHL-1. Western blot analysis failed to identify a band corresponding to the expected truncated polypeptide, suggesting it is not secreted or is rapidly degraded in plasma	Sanchez-Corral et al 2000
AMD	CFH	SCR3	575G>T	Cys192Phe	NA		Segregation analysis	Lower expression of FH and reduced secretion of the protein. and normal C3 in plasma of one carrier	Triebwasser et al., 2015; Wagner2016
AMD	CFH	SCR3	580G>A	Asp194Asn	NA				Raychaudhuri et al., 2011
aHUS/C3G	CFH	SCR3	592T>C	Trp198Arg	NA	Reported in aHUS			Szarvas et al, 2016
AMD	CFH	SCR3	593G>A	Trp198*	NA				Triebwasser et al., 2015
aHUS/C3G	CFH	SCR3	595A>G	Ser199Gly	NA	Reported in aHUS			Bu et al., 2014
AMD	CFH	SCR3	616G>A	Val206Met	NA				Triebwasser et al., 2015

Both	CFH	SCR4	647T>C	Ile216Thr	0.009	Reported in glomerulonephritis			Duvvari et al 2015; Dragon-Durey et al 2004; Gnappi et al 2012
AMD	CFH	SCR4	652G>T	Gly218*	NA	Reported in aHUS			Triebwasser et al., 2015
aHUS/C3G	CFH	SCR4	653G>A	Gly218Glu	NA	Reported in aHUS			Bresin et al, 2013; Fakhouri 2010
aHUS/C3G	CFH	SCR4	655T>C	Ser219Pro	NA				Zhan et al., 2013
aHUS/C3G	CFH	SCR4	670_672delAAG	Lys224Del	NA				Licht et al, 2006
aHUS/C3G	CFH	SCR4	694C>T	Arg232*	0.002	Reported in dense deposit disease			Servais et al 2012
AMD	CFH	SCR4	965G>A	Arg232Gln	NA				Triebwasser et al., 2015
AMD	CFH	SCR4	703T>C	Tyr235His	0.002		Case-control analysis (OR 3.417; p=0.44)		Fritsche et al., 2016; Zhan et al., 2013
AMD	CFH	SCR4	716T>C	Met239Thr	NA				Triebwasser et al., 2015
AMD	CFH	SCR4	770G>A	Arg257His	0.003				Triebwasser et al., 2015
Both	CFH	SCR4	773C>T	Pro258Leu	0.001	Reported in aHUS	Case-control analysis (OR 7.946; p=0.14)		Fritsche et al 2016; Saunders et al 2007
AMD	CFH	SCR4	790+1G>A	splicesite	NA		Segregation analysis	Lower expression of FH in three carriers and low C3 in plasma of one carrier	Triebwasser et al., 2015; Wagner2016
AMD	CFH	SCR5	833C>G	Tyr277*	NA			Lower expression of FH and normal C3 in plasma of one carrier	Triebwasser et al., 2015
AMD	CFH	SCR5	871A>T	Thr291Ser	NA		Case-control analysis (OR 0.373; p=0.54)		Zhan et al., 2013
AMD	CFH	SCR5	907C>T	Arg303Trp	0.007		Case-control analysis (OR 12.245; p=0.04)		Fritsche et al., 2016; Zhan et al., 2013
AMD	CFH	SCR5	942G>T	Trp314Cys	NA				Raychaudhuri et al., 2011
Both	CFH	SCR6	974G>A	Cys325Tyr	NA	Reported in aHUS			Triebwasser et al., 2015; Zhan et al., 2013; Maga 2010
aHUS/C3G	CFH	SCR6	996T>C	His332Tyr	NA				Haines et al 2005.
Both	CFH	SCR6	1022G>A	Arg341His	0.002	Reported in aHUS			Triebwasser et al., 2015; Zhan et al., 2013; Fakhouri 2010; Bresin 2013

Phenotype	Gene	Domain	c.	p.	ExAC (%)	aHUS/C3G comment	AMD comment	Functional Analyses	Source
AMD	CFH	SCR6	1032C>G	Tyr344*	NA				Zhan et al., 2013
aHUS/C3G	CFH	SCR6	1064A>C	Tyr355Ser	NA	Reported in aHUS			Jung et al 2011
AMD	CFH	SCR6	1091C>T	Pro364Leu	0.001				Triebwasser et al., 2015
AMD	CFH	SCR6	1135T>C	Trp379Arg	NA				Duvvari2015
AMD	CFH	SCR6	1139C>A	Ser380*	NA				Zhan et al., 2013
AMD	CFH	SCR6	1151C>G	Pro384Arg	NA				Triebwasser et al., 2015; Zhan et al., 2013
aHUS/C3G	CFH	SCR7	1160-2A>G	splice site	NA				Maga et al., 2010
AMD	CFH	SCR7	1189G>T	Gly397*	NA		Case-control analysis (OR 3.353; p=0.45)		Zhan et al., 2013
aHUS/C3G	CFH	SCR7	1189G>A	Gly397Arg	NA	Reported in aHUS			Bresin et al, 2013
AMD	CFH	SCR7	1228A>G	Lys410Glu	NA				Triebwasser et al., 2015
aHUS/C3G	CFH	SCR7	1231T>A	Ser411Thr	NA				Bresin et al, 2013
Both	CFH	SCR7	1291T>A	Cys431Ser	NA	Reported in glomerulonephritis		Lower expression of FH and normal C3 in plasma of one carrier	Triebwasser et al., 2015; Dragon-Durey 2004; Servais 2012
aHUS/C3G	CFH	SCR7	1292G>C	Cys431Ser	NA	Reported in aHUS			Dragon-Durey 2004
aHUS/C3G	CFH	SCR7	1292G>A	Cys431Tyr	NA	Reported in aHUS		absence in plasma of the factor H allele carrying the Cys431Tyr variant	Saunders et al., 2007; Montes et al 2008
AMD	CFH	SCR7	1310C>T	Ser437Phe	NA				Zhan et al., 2013
aHUS/C3G	CFH	SCR8	1343G>A	Cys448Tyr	NA				Szarvas et al, 2016
AMD	CFH	SCR8	1357A>G	Ile453Val	NA				Zhan et al., 2013
AMD	CFH	SCR8	1361A>C	Asp454Ala	NA				Triebwasser et al., 2015
AMD	CFH	SCR8	1404C>T	Ala468Ala	0.008		Case-control analysis (OR 5.368; p=0.29)		Fritsche et al., 2016
AMD	CFH	SCR8	1418C>T	Ala473Val	0.008		Case-control analysis (OR 0.929; p=0.87)		Fritsche et al., 2016; Triebwasser et al., 2015; Zhan et al., 2013
aHUS/C3G	CFH	SCR8	Not described	Lys474Asnfs*6	NA	Reported in aHUS			Bresin et al, 2013

aHUS/C3G	CFH	SCR8	1424A>C	Tyr475Ser	NA	Reported in aHUS			Nürnberger 2009
aHUS/C3G	CFH	SCR8	Not described	Leu479*	NA				Kavanagh et al, 2013
aHUS/C3G	CFH	SCR8	1494delA	Trp499Glyfs*17	NA			Truncated protein detected in plasma	Caprioli 2001
aHUS/C3G	CFH	SCR9	1553G>C	Arg518Thr	NA				Ault et al, 1997
aHUS/C3G	CFH	SCR9	1555A>G	Thr519Ala	NA				Haines et al 2005.
AMD	CFH	SCR9	1581G>C	Lys527Asn	NA		Case-control analysis (OR 3.279; p=0.46)		Zhan et al., 2013
aHUS/C3G	CFH	SCR9	1606T>C	Cys536Arg	NA				Ault et al, 1997
AMD	CFH	SCR9	1685C>A	Pro562His	NA		Case-control analysis (OR 9.533; p=0.06)		Triebwasser et al., 2015; Zhan et al., 2013
aHUS/C3G	CFH	SCR9	Not described	Cys564Pro	NA				Kavanagh et al, 2013
AMD	CFH	SCR10	1699A>G	Arg567Gly	0.001		Case-control analysis (OR 5.111; p=0.06)		Boon2008; Triebwasser et al., 2015
AMD	CFH	SCR10	1700G>A	Arg567Lys	NA		Case-control analysis (OR 0.383; p=0.56)		Zhan et al., 2013
aHUS/C3G	CFH	SCR10	1707C>A	Cys569*	NA	Reported in aHUS			Bresin et al, 2013
aHUS/C3G	CFH	SCR10	Not described	Leu578*	NA				Kavanagh et al, 2013
Both	CFH	SCR10	1745G>A	Arg582His	0.002	Reported in aHUS	Case-control analysis (OR 3.134; p=0.48)		Triebwasser et al., 2015; Zhan et al., 2013; Fremeaux-Bacchi 2013
aHUS/C3G	CFH	SCR10	1750A>T	Lys584*	NA	Reported in aHUS			Bresin et al., 2013
AMD	CFH	SCR10	1771G>A	Glu591Lys	NA				Zhan et al., 2013
AMD	CFH	SCR10	1825G>A	Val609Ile	0.030	Reported in aHUS	Case-control analysis (OR 0.704; p=0.29)	Affects FH expression and resulted in decreased alternative pathway activity and C3 level in remission	Fritsche et al., 2016; Triebwasser et al., 2015; Zhan et al., 2013; Maga er al 2010
aHUS/C3G	CFH	SCR10	1826T>A	Val609Asp	NA	Reported in aHUS			Szarvas et al, 2016
aHUS/C3G	CFH	SCR10	1832G>A	Cys611Tyr	NA	Reported in aHUS			Tschumi 2011
AMD	CFH	SCR10	1855G>A	Asp619Asn	NA				Triebwasser et al., 2015
aHUS/C3G	CFH	SCR10	1861C>A	Pro621Thr	NA	Reported in aHUS			Vaziri-Sani 2006

Phenotype	Gene	Domain	c.	p.	ExAC (%)	aHUS/C3G comment	AMD comment	Functional Analyses	Source
AMD	CFH	SCR10	1864A>T	Ile622Leu	NA		Case-control analysis (OR 3.328; p=0.45)		Zhan et al., 2013
aHUS/C3G	CFH	SCR10	1868G>C	Cys623Ser	NA	Reported in aHUS			Bresin et al, 2013; Sallée 2010
AMD	CFH	SCR10	1873G>T	Glu625*	0.001	Reported in aHUS	Case-control analysis (OR 0.18; p=0.20)		Fritsche et al., 2016; Maga et al 2010
aHUS/C3G	CFH	SCR11	1890T>G	Cys630Trp	NA	Reported in aHUS			Neumann et al., 2003
aHUS/C3G	CFH	SCR11	1905A>T	Glu635Asp	NA	Reported in aHUS			Kavanagh et al, 2013; Sellier-Leclerc 2007
aHUS/C3G	CFH	SCR11	1934insA	Lys646Glufs*8	NA	Reported in aHUS			Maga et al, 2010
AMD	CFH	SCR11	1998G>T	Lys666Asn	0.001		Case-control analysis (OR 2.591; p=0.34)		Triebwasser et al., 2015; Zhan et al., 2013
aHUS/C3G	CFH	SCR11	2009A>C	Lys670Thr	NA				Rodriguez de Cordoba et al, 2014
AMD	CFH	SCR11	2011A>T	Ile671Phe	NA				Zhan et al., 2013
aHUS/C3G	CFH	SCR11	2017T>A	Cys673Ser	NA	Reported in glomerulonephritis			Dragon-Durey 2004
aHUS/C3G	CFH	SCR11	2018G>A	Cys673Tyr	NA	Reported in aHUS			Dragon-Durey 2004
AMD	CFH	SCR12	2096A>G	His699Arg	NA				Triebwasser et al., 2015
aHUS/C3G	CFH	SCR12	2141C>G	Ser714*	NA	Reported in aHUS			Neumann et al., 2003
AMD	CFH	SCR12	2151C>A	Phe717Leu	0.007				Triebwasser et al., 2015
aHUS/C3G	CFH	SCR12	2165C>A	Ser722*	NA	Reported in aHUS			Szarvas et al, 2016
AMD	CFH	SCR12	2171C>A	Thr724Lys	0.010				Zhan et al., 2013
Both	CFH	SCR12	2195C>T	Thr732Met	0.010	Reported in glomerulosclerosis	Case-control analysis (OR 1.093; p=0.94)		Triebwasser et al., 2015; Zhan et al., 2013
aHUS/C3G	CFH	SCR12	2198G>A	Cys733Tyr	NA	Reported in aHUS			Kavanagh et al, 2013; Fremeaux-Bacchi 2013
aHUS/C3G	CFH	SCR13	2284G>T	Glu762*	NA	Reported in aHUS			Bresin et al, 2013
aHUS/C3G	CFH	SCR13	Not described	Lys768Lysfs*7	NA	Reported in aHUS			Bresin et al, 2013
aHUS/C3G	CFH	SCR13	2303_2304dupA	Lys769Glufs*6	NA				Dragon-Durey 2004
aHUS/C3G	CFH	SCR13	Not described	Asn774*	NA				Kavanagh et al, 2013

aHUS/C3G	CFH	SCR13	2339G>T	Arg780Ile	NA	Reported in aHUS		Saunders et al., 2007
aHUS/C3G	CFH	SCR13	Not described	Gly786fs*871	NA	Reported in aHUS		Bresin et al, 2013
AMD	CFH	SCR13	2405A>G	Asn802Ser	0.002		Case-control analysis (OR 0.108; p=0.06)	Fritsche et al., 2016
AMD	CFH	SCR14	2416G>A	Ala806Thr	NA			Raychaudhuri et al., 2011
AMD	CFH	SCR14	2461C>T	His821Tyr	0.009		Case-control analysis (OR 1.752; p=0.37)	Triebwasser et al., 2015; Zhan et al., 2013
aHUS/C3G	CFH	SCR14	Not described	Met823Thr	NA			Rodriguez de Cordoba et al, 2014
aHUS/C3G	CFH	SCR14	2503G>C	Val835Leu	NA			Saunders et al., 2007
aHUS/C3G	CFH	SCR14	2509G>A	Val837Ile	0.130	Reported in dense deposit disease		Zhang 2012; Matsumoto 2014
aHUS/C3G	CFH	SCR14	2540A>T	Glu847Val	NA	Reported in aHUS		Bresin et al, 2013
aHUS/C3G	CFH	SCR14	2548G>A	Glu850Lys	0.001	Reported in aHUS		Neumann et al., 2003
aHUS/C3G	CFH	SCR14	2557T>C	Cys853Arg	NA	Reported in aHUS		Hakobyan et al, 2010
aHUS/C3G	CFH	SCR14	Not described	Cys853Thr	NA	Reported in aHUS		Bresin et al, 2013
aHUS/C3G	CFH	SCR14	2591G>C	Cys864Ser	NA	Reported in aHUS		Bresin et al, 2013
aHUS/C3G	CFH	SCR15	2686_2700del	Lys896_Thr900del	NA			Caprioli et al, 2006
aHUS/C3G	CFH	SCR15	2608T>C	Cys870Arg	NA	Reported in aHUS		Stahl et al, 2008
aHUS/C3G	CFH	SCR15	2634C>T	His878His	1.150	Reported in aHUS		Neumann et al., 2003; Broeders 2014
aHUS/C3G	CFH	SCR15	2641A>C	Ile881Leu	NA	Reported in aHUS		Saunders et al., 2007
AMD	CFH	SCR15	2651C>A	Ser884Tyr	0.030		Case-control analysis (OR 3.218; p=0.47)	Triebwasser et al., 2015; Zhan et al., 2013
aHUS/C3G	CFH	SCR15	Not described	Glu889*	NA			Rodriguez de Cordoba et al, 2014
AMD	CFH	SCR15	2675C>T	Ala892Val	0.010		Case-control analysis (OR 1.596; p=0.47)	Fritsche et al., 2016; Triebwasser et al., 2015; Zhan et al., 2013
aHUS/C3G	CFH	SCR15	2678A>G	His893Arg	NA	Reported in aHUS		Dragon-Durey 2004
aHUS/C3G	CFH	SCR15	2695T>G	Tyr899Asp	NA	Reported in aHUS		Bresin et al, 2013
aHUS/C3G	CFH	SCR15	2697T>A	Tyr899*	NA	Reported in aHUS		Caprioli et al, 2006

Phenotype	Gene	Domain	c.	p.	ExAC (%)	aHUS/C3G comment	AMD comment	Functional Analyses	Source
aHUS/C3G	CFH	SCR15	2743T>A	Cys915Ser	NA	Reported in aHUS			Dragon-Durey 2004
aHUS/C3G	CFH	SCR15	2745C>A	Cys915*	NA	Reported in aHUS			Maga et al, 2010
aHUS/C3G	CFH	SCR15	2758T>C	Trp920Arg	NA	Reported in aHUS			Bresin et al, 2013; Noris 2010
aHUS/C3G	CFH	SCR15	2773C>T	Gln925*	NA	Reported in aHUS			Dragon-Durey 2004
aHUS/C3G	CFH	SCR15	2777G>T	Cys926Phe	NA	Reported in aHUS			Kavanagh et al, 2006
aHUS/C3G	CFH	SCR16	2851T>C	Tyr951His	0.001	Reported in aHUS			Caprioli 2003
aHUS/C3G	CFH	SCR16	2876G>A	Cys959Tyr	NA				Ault et al, 1997
aHUS/C3G	CFH	SCR16	2876G>C	Cys959Ser	NA	Reported in glomerulonephritis			Rusai et al., 2013
AMD	CFH	SCR16	2879T>C	Phe960Ser	0.005		Case-control analysis (OR 1.398; p=0.66)		Triebwasser et al., 2015; Zhan et al., 2013
aHUS/C3G	CFH	SCR16	2908A>G	Ile970Val	0.001	Reported in aHUS			Caprioli et al, 2006
Both	CFH	SCR16	2918G>A	Cys973Tyr	NA		Case-control analysis (OR 3.217; p=0.47)		Zhan et al., 2013; Goodship 2004
AMD	CFH	SCR16	2932T>C	Trp978Arg	NA				Triebwasser et al., 2015
aHUS/C3G	CFH	SCR16	2934G>T	Trp978Cys	NA	Reported in aHUS			Neumann et al., 2003
AMD	CFH	SCR16	2941C>T	Pro981Ser	NA				Triebwasser et al., 2015
aHUS/C3G	CFH	SCR17	2959A>G	Thr987Ala	NA				Saunders et al., 2007
aHUS/C3G	CFH	SCR17	2990A>C	Asn997Thr	NA	Reported in aHUS			Saunders et al., 2007
AMD	CFH	SCR17	3004G>C	Gly1002Arg	0.010		Case-control analysis (OR 0.436; p=0.20)		Fritsche et al., 2016; Triebwasser et al., 2015
aHUS/C3G	CFH	SCR17	Not described	Val1007Cys	NA	Reported in aHUS			Saunders et al, 2007
AMD	CFH	SCR17	3028G>A	Ala1010Thr	0.001				Zhan et al., 2013
aHUS/C3G	CFH	SCR17	3032delG	Gly1011Valfs*4	NA	Reported in aHUS			Bresin et al, 2013; Caprioli et al., 2006
aHUS/C3G	CFH	SCR17	3048C>A	Tyr1016*	0.001	Reported in aHUS			Bruel et al., 2017
aHUS/C3G	CFH	SCR17	3062A>T	Tyr1021Phe	NA				Neumann et al., 2003

AMD	CFH	SCR17	3110G>A	Trp1037*	NA			Triebwasser et al., 2015
aHUS/C3G	CFH	SCR17	3127T>C	Cys1043Arg	NA			Neumann et al., 2003
AMD	CFH	SCR18	3152C>T	Pro1051Leu	0.003		Case-control analysis (OR 0.87; p=0.88)	Fritsche et al., 2016; Triebwasser et al., 2015
AMD	CFH	SCR18	3168T>G	Asn1056Lys	0.007		Case-control analysis (OR 0.082; p=0.03)	Fritsche et al., 2016; Zhan et al., 2013
aHUS/C3G	CFH	SCR18	3179T>C	Val1060Ala	NA	Reported in aHUS		Guigonis et al, 2005
aHUS/C3G	CFH	SCR18	3231T>G	Cys1077Trp	NA	Reported in aHUS		Cho et al, 2007
AMD	CFH	SCR18	3265_3266insA	Val1089Aspfs*28	NA			Raychaudhuri et al., 2011
aHUS/C3G	CFH	SCR19	3350A>G	Asn1117Ser	NA	Reported in glomerulonephritis		Fervenza et al.,2012
aHUS/C3G	CFH	SCR19	3355G>A	Asp1119Asn	NA	Reported in aHUS		Bresin et al, 2013
aHUS/C3G	CFH	SCR19	3356A>G	Asp1119Gly	NA			Richards 2001
aHUS/C3G	CFH	SCR19	3389C>T	Pro1130Leu	NA			Kavanagh et al, 2013; Fremeaux-Bacchi 2013
aHUS/C3G	CFH	SCR19	3401T>G	Val1134Gly	NA	Reported in aHUS		Neumann et al., 2003
aHUS/C3G	CFH	SCR19	3405G>C	Glu1135Asp	NA	Reported in aHUS		Sullivan et al., 2010
aHUS/C3G	CFH	SCR19	Not described	Glu1135Arg	NA			Kavanagh et al, 2013
aHUS/C3G	CFH	SCR19	3409C>T	Gln1137*	NA	Reported in aHUS		Kavanagh et al, 2013
aHUS/C3G	CFH	SCR19	3410A>T	Gln1137Leu	NA	Reported in aHUS		Bresin et al, 2013; Noris et al., 2010
aHUS/C3G	CFH	SCR19	3415C>T	Gln1139*	NA	Reported in aHUS		Cho et al, 2007
aHUS/C3G	CFH	SCR19	3424T>G	Tyr1142Asp	NA	Reported in aHUS		Neumann et al., 2003
aHUS/C3G	CFH	SCR19	3425A>G	Tyr1142Cys	NA	Reported in aHUS		Saunders 2007; Morgan et al., 2011
aHUS/C3G	CFH	SCR19	3445C>T	Arg1149*	NA	Reported in aHUS		Szilágyi et al., 2013
aHUS/C3G	CFH	SCR19	3454T>A	Cys1152Ser	NA	Reported in aHUS		Sevinc et al, 2015
aHUS/C3G	CFH	SCR19	3463G>T	Gly1155*	NA	Reported in aHUS		Habbig et al., 2016
aHUS/C3G	CFH	SCR19	3469T>C	Trp1157Arg	NA	Reported in aHUS		Neumann et al., 2003
aHUS/C3G	CFH	SCR19	3481C>A	Pro1161Thr	NA	Reported in aHUS		Szarvas et al, 2016

Phenotype	Gene	Domain	c.	p.	ExAC (%)	aHUS/C3G comment	AMD comment	Functional Analyses	Source
aHUS/C3G	CFH	SCR19	3489C>G	Cys1163Trp	NA	Reported in aHUS			Caprioli 2003
aHUS/C3G	CFH	SCR19	3493C>T	His1165Tyr	0.002	Reported in aHUS			Lopes et al., 2015
aHUS/C3G	CFH	SCR19	3493+1G>A	splice site	NA	Reported in aHUS			Neumann et al., 2003
Both	CFH	SCR19	3497C>A	Pro1166Leu	NA	Reported in aHUS			Zhan et al., 2013; Bresin et al, 2013
aHUS/C3G	CFH	SCR20	3503T>A	Val1168Glu	NA	Reported in aHUS			Stahl et al, 2008; Kavanagh et al, 2013
aHUS/C3G	CFH	SCR20	3505A>C	Ile1169Leu	NA	Reported in aHUS			
aHUS/C3G	CFH	SCR20	3514G>T	Glu1172*	0.001	Reported in aHUS		Mutant protein has reduced binding to heparin, C3b/ C3d and endothelial cells. Heparin affinity chromatography revealed reduced binding of mutant protein to heparin and surface plasmon resonance studies showed impaired binidng to C3b and C3d.	Caprioli 2003; Manuelian 2003; Heinen et al 2006
aHUS/C3G	CFH	SCR20	3530A>G	Tyr1177Cys	NA	Reported in aHUS			Kavanagh et al, 2013; Besbas et al., 2013
aHUS/C3G	CFH	SCR20	3546G>C	Arg1182Ser	NA	Reported in aHUS			Jokiranta et al., 2006
aHUS/C3G	CFH	SCR20	3548G>T	Trp1183Leu	NA				Dragon-Durey 2004
aHUS/C3G	CFH	SCR20	3549G>A	Trp1183*	NA	Reported in aHUS			Saunder et al., 2007
aHUS/C3G	CFH	SCR20	3549G>T	Trp1183Cys	NA	Reported in aHUS			Maga et al, 2010
aHUS/C3G	CFH	SCR20	3550A>G	Thr1184Ala	NA	Reported in aHUS			Bresin et al, 2013; Wilson et al., 2011
aHUS/C3G	CFH	SCR20	3551C>G	Thr1184Arg	NA				Richards et al., 2001
aHUS/C3G	CFH	SCR20	3557A>C	Lys1186Thr	NA	Reported in aHUS			Le Quintrec et al, 2008
aHUS/C3G	CFH	SCR20	Not described	Lys1188del	NA	Reported in aHUS			Bresin et al, 2013

aHUS/C3G	CFH	SCR20	3565C>T	Leu1189Phe	NA	Reported in aHUS		Slightly increased affinity for C3b and heparin	Esparza-Gordillo et al., 2005; Ferreira et al., 2009
aHUS/C3G	CFH	SCR20	3566T>G	Leu1189Arg	NA				Perez-Caballero 2001
aHUS/C3G	CFH	SCR20	3566T>C	Leu1189Pro	NA	Reported in aHUS			Kavanagh et al., 2013
aHUS/C3G	CFH	SCR20	3572C>G	Ser1191Trp	NA	Reported in aHUS			Rodriguez de Cordoba et al., 2004; Bresin et al., 2013; Ferreira et al., 2009
aHUS/C3G	CFH	SCR20	3583G>T	Glu1195*	NA	Reported in aHUS			Maga et al, 2010
aHUS/C3G	CFH	SCR20	3592G>A	Glu1198Lys	NA	Reported in aHUS			Vaziri-Sani et al., 2006
aHUS/C3G	CFH	SCR20	3592G>T	Glu1198*	NA	Reported in aHUS			Saunders et al., 2007; Stahl et al., 2008
aHUS/C3G	CFH	SCR20	3593A>C	Glu1198Ala	NA	Reported in aHUS			Caprioli et al., 2003
aHUS/C3G	CFH	SCR20	3596T>C	Phe1199Ser	NA	Reported in aHUS			Dragon-Durey 2004
aHUS/C3G	CFH	SCR20	3598G>T	Val1200Leu	NA	Reported in aHUS			Bresin et al, 2013; Noris et al., 2010
AMD	CFH	SCR20	3606A>C	Lys1202Asn	NA		Case-control analysis (OR 2.463; p=0.41)		Zhan et al., 2013
Both	CFH	SCR20	3607C>T	Arg1203Trp	0.008	Reported in aHUS	Case-control analysis (OR 0.676; p=0.82)	No Lysis in hemolymtic assay	Fritsche et al., 2016; Szarvas2016; Westra 2010; Szarvas et al, 2016; Kavanagh et al, 2013; Westra et al., 2010
aHUS/C3G	CFH	SCR20	3611G>A	Gly1204Glu	NA	Reported in aHUS			Saunders et al., 2007
AMD	CFH	SCR20	3617G>A	Arg1206His	NA				Triebwasser et al., 2015
aHUS/C3G	CFH	SCR20	3620T>G	Leu1207Arg	NA	Reported in aHUS			Saunders et al., 2007
aHUS/C3G	CFH	SCR20	3632C>T	Ser1211Phe	NA	Reported in aHUS			Saunders er al., 2007
aHUS/C3G	CFH	SCR20	3643C>G	Arg1215Gly	NA	Reported in aHUS			Warwicker et al., 1998
aHUS/C3G	CFH	SCR20	3643C>T	Arg1215*	0.001	Reported in aHUS			Fremeaux-Bacchi et al., 2013
aHUS/C3G	CFH	SCR20	3652T>C	Cys1218Arg	NA	Reported in aHUS			Bresin et al, 2013; Hakobyan et al., 2010

Phenotype	Gene	Domain	c.	p.	ExAC (%)	aHUS/C3G comment	AMD comment	Functional Analyses	Source
aHUS/C3G	CFH	SCR20	3676C>T	Pro1226Ser	NA	Reported in aHUS		Reduced heparin, C3b, C3d, and endothelial cell binding	Neumann et al., 2003; Józsi et al., 2006; Jokiranta et al., 2006
AMD	CFH	SCR20	3680C>T	Thr1227Ile	0.002				Triebwasser et al., 2015
aHUS/C3G	CFH	SCR20	3646_3648delACA	Thr1216Del	NA				Neumann et al., 2003
aHUS/C3G	CFH	SCR20	3695_3698delAGAA	Deletion removes stop codon	NA	Reported in aHUS		This mutation deletes the termination codon and results in a longer polypeptide chain	Neumann et al., 2003
aHUS/C3G	CFH	SCR20	3677_*4del24	Deletion Removes stop codon	NA	Reported in aHUS		Functional analysis demonstrate mutant protein is expressed and synthesised but is not transported normally from the cell	Caprioli et al., 2001; Buddles et al 2000; Ying et al., 1999

Phenotype	Gene	Domain	c.	p.	ExAC (%)	aHUS/C3G comment	AMD comment	Functional Analyses	Source
AMD	CFI	Terminus	58A>G	Val20Ile	NA				Kavanagh et al., 2015
AMD	CFI	FIMAC	119A>G	His40Arg	0.002				Kavanagh et al., 2015; Zhan et al., 2013
aHUS/C3G	CFI	FIMAC	128G>T	Cys43Phe	NA	Reported in aHUS			Bienaime et al., 2010; Nilsson et al., 2010
AMD	CFI	FIMAC	130G>A	Asp44Asn	0.002				Kavanagh et al., 2015; Zhan et al., 2013
Both	CFI	FIMAC	148C>G	Pro50Ala	0.010	Reported in aHUS		Elevated FB in plasma; normal C3 and FI in plasma; impaired function towards degradation of the alpha-chains of C4b and C3b in solution when FH was used as cofactor	Bienaime et al., 2010; Bresin et al., 2013; Nilsson et al., 2010; Kavanagh et al., 2015
AMD	CFI	FIMAC	160T>C	Cys54Arg	NA				Kavanagh et al., 2015; Zhan et al., 2013
AMD	CFI	FIMAC	162C>A	Cys54*	NA				Kavanagh et al., 2015
AMD	CFI	FIMAC	163A>T	Ile55Phe	NA				Zhan et al., 2013
Both	CFI	FIMAC	191C>T	Pro64Leu	0.020	Reported in aHUS		Hypoactive complement factor I in in vivo assay of retinal vascularization in zebrafish (enlarged hyaloid vessels),	Kavanagh et al., 2015; Tan et al., 2017; Maga et al., 2010; Seddon et al., 2013
AMD	CFI	FIMAC	209A>C	Asn70Thr	0.005				Zhan et al., 2013
aHUS/C3G	CFI	FIMAC	215C>G	Thr72Ser	0.00082	Reported in aHUS			Noris et al., 2010
AMD	CFI	FIMAC	245T>C	Phe82Ser	NA				Zhan et al., 2013
aHUS/C3G	CFI	FIMAC	248C>A	Pro83Gln	NA	Reported in aHUS			Szarvas et al., 2016
aHUS/C3G	CFI	FIMAC	269G>A	Ser90Asn	NA	Reported in glomerulonephritis			Sellier-Leclerc et al, 2007; Le Quintec et al., 2008
AMD	CFI	FIMAC	316T>C	Cys106Arg	0.001				Zhan et al., 2013
AMD	CFI	FIMAC	319A>G	Thr107Ala	0.040				Zhan et al., 2013
AMD	CFI	FIMAC	326A>C	Glu109Ala	NA				Kavanagh et al., 2015
aHUS/C3G	CFI	SRCR	353A>G	His118Arg	NA	Reported in aHUS			Bresin et al., 2013

Phenotype	Gene	Domain	c.	p.	ExAC (%)	aHUS/C3G comment	AMD comment	Functional Analyses	Source
AMD	CFI	SRCR	373G>A	Gly125Arg	NA			Hypoactive complement factor I in in vivo assay of retinal vascularization in zebrafish (enlarged hyaloid vessels)	Kavanagh et al., 2015; Zhan et al., 2013; Tan et al., 2017
AMD	CFI	SRCR	380T>C	Val127Ala	NA		Case-control analysis (OR 3.374; p=0.45)		Kavanagh et al., 2015; Zhan et al., 2013
AMD	CFI	SRCR	386T>G	Val129Gly	NA		Case-control analysis (OR 0.352; p=0.52)		Fritsche et al., 2016; Zhan et al., 2013
aHUS/C3G	CFI	SRCR	412A>G	Met138Val	NA	Reported in aHUS			Nilsson et al., 2010
aHUS/C3G	CFI	SRCR	414G>T	Met138Ile	0.00082	Reported in aHUS			Kavanagh et al., 2008
aHUS/C3G	CFI	SRCR	434G>A	Trp145*	NA	Reported in aHUS			Kavanagh et al., 2005; Nilsson et al., 2010
AMD	CFI	SRCR	485G>A	Gly162Asp	0.001		Case-control analysis (OR 20.291; p=0.002)		Fritsche et al., 2016; Kavanagh et al., 2015; Zhan et al., 2013
aHUS/C3G	CFI	SRCR	491A>T	Asp164Val	NA	Reported in aHUS			Sullivan et al., 2010
AMD	CFI	SRCR	530A>T	Asn177Ile	0.005	Reported in aHUS			Kavanagh et al., 2015; Bresin et al., 2013; Seddon et al., 2013
Both	CFI	SRCR	548A>G	His183Arg	0.060	Reported in aHUS	Case-control analysis (OR 2.578; p=0.56)		Fritsche et al., 2016; Sellier-Leclerc et al., 2007; Bresin et al., 2013
AMD	CFI	SRCR	550G>A	Val184Met	NA				Kavanagh et al., 2015
AMD	CFI	SRCR	559C>T	Arg187*	0.002		Case-control analysis (OR 13.628; p=0.02)		Fritsche et al., 2016; Zhan et al., 2013
AMD	CFI	SRCR	560G>A	Arg187Gln	0.010		Case-control analysis (OR 1.304; p=0.69)	Normal FI in vivo assay of retinal vascularization in zebrafish.	Fritsche et al., 2016; Kavanagh et al., 2015; Tan et al., 2017
AMD	CFI	SRCR	594T>C	Phe198Leu	NA				Kavanagh et al., 2015
AMD	CFI	SRCR	605G>T	Arg202Ile	NA			This variant had no effect on FI levels in human serum	Kavanagh et al., 2015
AMD	CFI	SRCR	608C>T	Thr203Ile	0.050		Case-control analysis (OR 2.462; p=0.03)	Normal FI in vivo assay of retinal vascularization in zebrafish.	Fritsche et al., 2016; Kavanagh et al., 2015; Zhan et al., 2013; Tan et al., 2017
AMD	CFI	SRCR	206T>A	Tyr206Asn	0.010				Kavanagh et al., 2015

AMD	CFI	LA1	651G>C	Gln217His	NA				Kavanagh et al., 2015
AMD	CFI	LA1	662C>A	Ser221Tyr	NA				Kavanagh et al., 2015
AMD	CFI	LA1	670G>A	Asp224Asn	NA				Kavanagh et al., 2015
AMD	CFI	LA1	688G>A	Val230Met	NA				Kavanagh et al., 2015
aHUS/C3G	CFI	LA1	689T>A	Val230Glu	NA	Reported in aHUS			Bresin et al., 2013
aHUS/C3G	CFI	LA1	739T>G	Cys247Gly	NA	Reported in aHUS			Saunders et al., 2007; Bresin et al., 2013; Kavanagh et al., 2015
AMD	CFI	LA1	743G>A	Gly248Glu	NA				Kavanagh et al., 2015
AMD	CFI	LA1	747C>A	Asp249Glu	NA			Hypoactive complement factor I in in vivo assay of retinal vascularization in zebrafish (enlarged hyaloid vessels)	Tan et al., 2017
AMD	CFI	LA2	788G>T	Gly263Val	NA		Case-control analysis (OR 1.917; p=0.43)		Fritsche et al., 2016; Kavanagh et al., 2015; Zhan et al., 2013
AMD	CFI	LA2	801A>C	Lys267Asn	NA				Zhan et al., 2013
AMD	CFI	LA2	839G>A	Gly280Asp	NA				Kavanagh et al., 2015
Both	CFI	LA2	859G>A	Gly287Arg	0.003	Reported in aHUS	Case-control analysis (OR 4.605; p=0.008)	Normal FI in vivo assay of retinal vascularization in zebrafish.	Fritsche et al., 2016; Kavanagh et al., 2015; Tan et al., 2017; Maga et al., 2010; Seddon et al., 2013
AMD	CFI	LA2	870A>C	Glu290Asp	0.002			Hypoactive complement factor I in in vivo assay of retinal vascularization in zebrafish (enlarged hyaloid vessels).	Tan et al., 2017
aHUS/C3G	CFI	linker region	904+1G>A	splice site	NA	Reported in aHUS			Zhang et al., 2016
AMD	CFI	linker region	907G>A	Glu303Lys	0.001			Hypoactive complement factor I in in vivo assay of retinal vascularization in zebrafish (enlarged hyaloid vessels).	Tan et al., 2017
AMD	CFI	linker region	913G>T	Glu305*	NA			Hypoactive complement factor I in in vivo assay of retinal vascularization in zebrafish (enlarged hyaloid vessels)	Tan et al., 2017

Phenotype	Gene	Domain	c.	p.	ExAC (%)	aHUS/C3G comment	AMD comment	Functional Analyses	Source
aHUS/C3G	CFI	linker region	917T>G	Ile306Ser	NA	Reported in glomerulonephritis			Leroy et al., 2011; Servais et al., 2012
AMD	CFI	linker region	930C>A	Asp310Glu	NA			Hyperactive complement factor I in in vivo assay of retinal vascularization in zebrafish (smaller hyaloid vessels).	Kavanagh et al., 2015; Tan et al., 2017
Both	CFI	linker region	949C>T	Arg317Trp	0.010	Reported in aHUS	Case-control analysis (OR 12.198; p<0.001)	Normal FI plasma level and normal functioning on hemolytic assay; only impaired secretion compared to wildtype FI	Fritsche et al., 2016; Kavanagh et al., 2015; Zhan et al., 2013; Caprioli et al., 2006; Nilsson et al., 2010; Seddon et al., 2013
AMD	CFI	linker region	950G>A	Arg317Gln	0.001		Case-control analysis (OR 2.807; p=0.52)		Fritsche et al., 2016; Kavanagh et al., 2015; Zhan et al., 2013
aHUS/C3G	CFI	linker region	979T>C	Cys327Arg	0.00082	Reported in glomerulonephritis			Servais et al., 2012
AMD	CFI	linker region	982G>A	Gly328Arg	0.010		Case-control analysis (OR 5.669; p=0.22)		Fritsche et al., 2016; Kavanagh et al., 2015; Zhan et al., 2013
AMD	CFI	linker region	1015C>T	Arg339*	0.002		Case-control analysis (OR 9.634; p=0.06)		Fritsche et al., 2016
Both	CFI	SP	1025G>A	Gly342Glu	NA	Reported in aHUS	Case-control analysis (OR 3.38; p=0.45)		Zhan et al., 2013; Fremeaux-Bacchi et al., 2013
aHUS/C3G	CFI	SP	1034G>A	Arg345Gln	0.00082	Haemolysis, elevated liver enzymes & low platelet count			Fakhouri et al., 2008
aHUS/C3G	CFI	SP	1045G>A	Gly349Arg	NA	Reported in aHUS			Noris et al., 2010
AMD	CFI	SP	1063G>A	Val355Met	NA		Case-control analysis (OR 2.541; p=0.35)		Kavanagh et al., 2015; Zhan et al., 2013
AMD	CFI	SP	1066G>C	Ala356Pro	0.002				Kavanagh et al., 2015
Both	CFI	SP	1071T>G	Ile357Met	0.003	Reported in aHUS	Case-control analysis (OR 9.389; p=0.07)		Fritsche et al., 2016; Nilsson et al., 2008; Bresin et al., 2013; Geerdink et al., 2012
AMD	CFI	SP	1085G>C	Gly362Ala	0.001				Kavanagh et al., 2015

Both	CFI	SP	1106A>C	Tyr369Ser	0.001	Reported in aHUS		Normal FH and C4 levels; low C3 levels in serum	Kavanagh et al., 2015; Chan et al., 2009; Maga et al., 2010; Seddon et al., 2013
AMD	CFI	SP	1122G>T	Trp374Cys	NA				Kavanagh et al., 2015
AMD	CFI	SP	1166G>A	Arg389His	0.002				Kavanagh et al., 2015
Both	CFI	SP	1195T>C	Trp399Arg	0.002	Reported in aHUS			Kavanagh et al., 2015; Noris et al., 2010; Seddon et al., 2013
aHUS/C3G	CFI	SP	1204C>T	Pro402Ser	NA	Haemolysis, elevated liver enzymes & low platelet count			Crovetto et al., 2012
Both	CFI	SP	1207G>A	Asp403Asn	0.002	Reported in aHUS	Case-control analysis (OR 1.693; p=0.63)		Fritsche et al., 2016; Kavanagh et al., 2015; Zhan et al., 2013; Fremeaux-Bacchi et al., 2013; Seddon et al., 2013
Both	CFI	SP	1216C>T	Arg406Cys	0.006	Reported in aHUS	Case-control analysis (OR 1.051; p=0.96)		Fritsche et al., 2016; Moore et al., 2010
AMD	CFI	SP	1233C>A	Tyr411*	NA		Case-control analysis (OR 1.828; p=0.33)		Fritsche et al., 2016
Both	CFI	SP	1234G>A	Val412Met	0.010	Reported in aHUS	Segregation analysis		Alberti et al., 2013; Pras et al., 2015
Both	CFI	SP	1246A>C	Ile416Leu	0.110	Reported in aHUS	Case-control analysis (OR 2.227; p=0.47)	Low FI and C3 serum levels; normal FB levels	Fritsche et al., 2016; Kavanagh et al., 2015; Bienaime et al., 2010; Sellier-Leclerc et al., 2007; Le Quintrec et al., 2008
AMD	CFI	SP	1253A>T	His418Leu	0.001			Homozygous variation results in FI deficiency (low or undetectable FI and C3 levels)	Kavanagh et al., 2015; Vyse et al., 1996
aHUS/C3G	CFI	SP	1271G>A	Gly424Asp	NA	Reported in aHUS			Fakhouri et al., 2010
AMD	CFI	SP	1287C>G	Asp429Glu	NA		Case-control analysis (OR 3.361; p=0.45)		Zhan et al., 2013
Both	CFI	SP	1291G>A	Ala431Thr	0.002	Reported in aHUS			Zhan et al., 2013; Bienaime et al., 2010

Phenotype	Gene	Domain	c.	p.	ExAC (%)	aHUS/C3G comment	AMD comment	Functional Analyses	Source
aHUS/C3G	CFI	SP	1297A>G	Ile433Val	NA	Reported in aHUS			Sellier-Leclerc et al., 2007; Bienaime et al., 2010
aHUS/C3G	CFI	SP	1367G>T	Trp456Leu	NA	Reported in aHUS			Bienaime et al., 2010
AMD	CFI	SP	1386A>T	Gln462His	0.010		Case-control analysis (OR 0.595; p=0.53)		Fritsche et al., 2016; Kavanagh et al., 2015
AMD	CFI	SP	1399T>C	Cys467Arg	NA				Kavanagh et al., 2015
Both	CFI	SP	1420C>T	Arg474*	0.005	Reported in aHUS	Case-control analysis (OR 1.065; p=0.95)	Low FI and C3 serum levels; normal FB levels	Fritsche et al., 2016; Kavanagh et al., 2015; Bienaime et al., 2010; Fremeaux-Bacchi et al., 2004; Nilsson et al., 2010; Tan et al., 2017
Both	CFI	SP	1421G>A	Arg474Gln	0.002	Reported in aHUS	Case-control analysis (OR 4.056; p=0.12)	Normal FI protein level	Fritsche et al., 2016; Kavanagh et al., 2015; Vyse et al., 1996; Szarvas et al., 2016
AMD	CFI	SP	1429G>C	Asp477His	0.003		Case-control analysis (OR 10.258; p=0.05)		Fritsche et al., 2017
Both	CFI	SP	1429+1C>G	Splicesite	0.003	Reported in aHUS			Kavanagh et al., 2015; Zhan et al., 2013; Bresin et al., 2013; Seddon et al., 2013
AMD	CFI	SP	1459G>T	Gly487Cys	NA				Kavanagh et al., 2015
AMD	CFI	SP	1474A>C	Ile492Leu	0.002				Kavanagh et al., 2015
AMD	CFI	SP	1498G>A	Gly500Arg	0.003				Kavanagh et al., 2015
AMD	CFI	SP	1504C>T	Arg502Cys	NA				Kavanagh et al., 2015
AMD	CFI	SP	1534G>A	Gly512Ser	0.002		Case-control analysis (OR 5.485; p=0.23)		Fritsche et al., 2016; Kavanagh et al., 2015; Zhan et al., 2013
aHUS/C3G	CFI	SP	1555G>A	Asp519Asn	0.00166	Reported in aHUS			Caprioli et al., 2006; Nilsson et al., 2010
aHUS/C3G	CFI	SP	1565A>C	Lys522Thr	NA	Reported in aHUS			Maga et al., 2010
aHUS/C3G	CFI	SP	1571A>T	Asp524Val	NA	Reported in aHUS			Fremeaux-Bacchi et al., 2004; Bresin et al., 2013

AMD	CFI	SP	1594A>G	Met532Val	NA		Normal FI in vivo assay of retinal vascularization in zebrafish.	Tan et al., 2017
AMD	CFI	SP	1608T>A	Asn536Lys	NA			Kavanagh et al., 2015
AMD	CFI	SP	1622G>A	Trp541*	NA			Kavanagh et al., 2015
AMD	CFI	SP	1624G>A	Gly542Ser	0.001			Zhan et al., 2013
AMD	CFI	SP	1628T>C	Val543Ala	NA			Kavanagh et al., 2015
aHUS/C3G	CFI	SP	1637G>A	Trp546*	NA	Reported in aHUS		Fremeaux-Bacchi et al., 2004; Nilsson et al., 2010
Both	CFI	SP	1661A>T	Glu554Val	0.002	Reported in aHUS		Kavanagh et al., 2015; Noris et al., 2010; Bresin et al., 2013; Seddon et al., 2013
AMD	CFI	SP	1733T>C	Ile578Thr	0.002			Kavanagh et al., 2015; Zhan et al., 2013
AMD	CFI	SP	1738C>T	Gln580*	0.001			Kavanagh et al., 2015

Phenotype	Gene	Domain	c.	p.	ExAC (%)	aHUS/C3G comment	AMD comment	Functional Analyses	Source
AMD	C3	MG1	181G>A	Asp61Asn	0.002		Case-control analysis (OR 5.30; p=0.24)		Fritsche et al., 2016; Zhan et al., 2013
aHUS/C3G	C3	MG1	188C>T	Pro63Leu	NA	Reported in aHUS			Schramm et al., 2015
aHUS/C3G	C3	MG1	219G>T	Lys73Asn	NA	Reported in aHUS			Finn et al., 1994; Abrera-Abeleda et al., 2011; Schramm et al., 2015
aHUS/C3G	C3	MG1	310A>G	Lys104Glu	NA	Reported in aHUS			Szarvas et al., 2016
aHUS/C3G	C3	MG2	485C>A	Thr162Lys	NA	Reported in aHUS			Noris et al., 2010; Schramm et al., 2015
aHUS/C3G	C3	MG2	553C>G	Gln185Glu	NA	Reported in aHUS			Noris et al., 2010; Schramm et al., 2015
AMD	C3	MG2	562G>A	Val188Ile	0.005		Case-control analysis (OR 3.84; p=0.13)		Fritsche et al., 2016; Zhan et al., 2013
AMD	C3	MG3	769G>A	Ala257Thr	0.005				Zhan et al., 2013
aHUS/C3G	C3	MG3	784G>T	Gly262Trp	NA	Reported in aHUS			Sánchez Chinchilla et al., 2014
AMD	C3	MG3	890C>T	Ser297Leu	0.002				Zhan et al., 2013
AMD	C3	MG3	928G>A	Gly310Arg	0.005		Case-control analysis (OR 0.15; p=0.15)		Fritsche et al., 2016
AMD	C3	MG3	991A>C	Ile331Leu	NA		Case-control analysis (OR 0.36; p=0.53)		Fritsche et al., 2016; Zhan et al., 2013
aHUS/C3G	C3	MG3	1028G>A	Arg343His	0.002	Reported in aHUS			Zhang et al., 2016
AMD	C3	MG3	1042A>G	Ile348Val	NA		Case-control analysis (OR 0.37; p=0.28)		Fritsche et al., 2016
AMD	C3	MG4	1262G>A	Ser421Asn	NA				Zhan et al., 2013
Both	C3	MG4	1273C>T	Arg425Cys	0.017	Reported in aHUS			Zhan et al., 2013; Fan et al., 2013; Cho et al., 2016; Matsukuma et al., 2014
AMD	C3	MG5	1411C>T	Leu471Phe	NA		Case-control analysis (OR 3.13; p=0.48)		Fritsche et al., 2016; Zhan et al., 2013
aHUS/C3G	C3	MG5	1433G>T	Arg478Leu	0.004	Reported in aHUS			Noris et al., 2010; Schramm et al., 2015
aHUS/C3G	C3	MG5	1601A>T	Tyr534Phe	NA	Reported in aHUS			Bresin et al., 2013

AMD	C3	MG5	1618G>T	Ala540Ser	0.006		Case-control analysis (OR 0.66; p=0.61)	Fritsche et al., 2016
AMD	C3	MG5	1624G>A	Gly542Ser	0.002		Case-control analysis (OR 3.17; p=0.47)	Fritsche et al., 2016
aHUS/C3G	C3	MG6	1685C>T	Ser562Leu	0.008	Reported in aHUS		Fan et al., 2013; Schramm et al., 2015
AMD	C3	MG6	1702G>A	Gly568Ser	0.001			Zhan et al., 2013
AMD	C3	MG6	1742T>C	Met581Thr	NA			Zhan et al., 2013
aHUS/C3G	C3	MG6	1775G>A	Arg592Gln	NA	Reported in aHUS		Fremeaux-Bacchi et al., 2008; Schramm et al., 2015
aHUS/C3G	C3	LNK	1807T>G	Phe603Val	NA	Reported in aHUS		Maga et al., 2010; Schramm et al., 2015
AMD	C3	LNK	1819A>G	Lys607Glu	0.002		Case-control analysis (OR 0.75; p=0.74)	Fritsche et al., 2016; Zhan et al., 2013
AMD	C3	LNK	1873A>T	Ile625Phe	0.046		Case-control analysis (OR 0.67; p=0.44)	Fritsche et al., 2016
Both	C3	LNK	1898A>G	Lys633Arg	0.040	Reported in aHUS	Case-control analysis (OR 1.09; p=0.77)	Fritsche et al., 2016; Zhan et al., 2013; Seddon et al., 2013; Schramm et al., 2015
AMD	C3	LNK	1909G>C	Gly637Arg	0.022			Zhan et al., 2013
AMD	C3	LNK	1988C>G	Pro663Arg	0.003		Case-control analysis (OR 1.07; p=0.92)	Fritsche et al., 2016
AMD	C3	ANA	2104C>T	Pro702Ser	0.001		Case-control analysis (OR 3.24; p=0.47)	Fritsche et al., 2016; Zhan et al., 2013
AMD	C3	ANA	2107A>T	Met703Leu	NA		Case-control analysis (OR 0.35; p=0.52)	Fritsche et al., 2016; Zhan et al., 2013
AMD	C3	ANA	2126G>A	Arg709His	NA		Case-control analysis (OR 3.16; p=0.48)	Zhan et al., 2013; Bresin et al., 2013; Duvvari et al., 2014

Phenotype	Gene	Domain	c.	p.	ExAC (%)	aHUS/C3G comment	AMD comment	Functional Analyses	Source
Both	C3	ANA	2203C>T	Arg735Trp	NA	Reported in aHUS	Case-control analysis (OR 0.99; p=0.95)	This variant showed no functional effects on MCP binding, FI cofactor activity, FB binding, CR1 binding and FH binding.	Fritsche et al., 2016; Zhan et al., 2013; Seddon et al., 2013; Duvvari et al., 2014; Brackman et al., 2011; Fremeaux-Bacchi et al., 2008
AMD	C3	ANA	2207G>A	Arg736Gln	0.005		Case-control analysis (OR 0.60; p=0.47)		Fritsche et al., 2016; Zhan et al., 2013
Both	C3	αNT	2284G>A	Val762Ile	0.002	Reported in aHUS	Case-control analysis (OR 3.33; p=0.45)		Fritsche et al., 2016; Noris et al., 2010
aHUS/C3G	C3	αNT	2562C>G	Tyr854*	NA	Reported in aHUS			Fremeaux-Bacchi et al., 2008; Schramm et al., 2015
AMD	C3	MG7	2564G>A	Arg855Gln	0.006		Case-control analysis (OR 0.36; p=0.15)		Fritsche et al., 2016
AMD	C3	MG7	2609C>T	Pro870Leu	NA				Zhan et al., 2013
AMD	C3	MG7	2633C>A	Thr878Asn	NA		Case-control analysis (OR 0.35; p=0.52)		Fritsche et al., 2016; Zhan et al., 2013
aHUS/C3G	C3	MG7	2852G>A	Arg951His	0.003	Reported in aHUS			Szarvas et al., 2016
Both	C3	TED	3023C>T	Ser1008Leu	0.004	Reported in aHUS	Case-control analysis (OR 3.27; p=0.2)		Fritsche et al., 2016; Zhan et al., 2013; Phillips et al., 2016
AMD	C3	TED	3049G>C	Gly1017Arg	0.001				Zhan et al., 2013
aHUS/C3G	C3	TED	3085G>A	Asp1029Asn	NA	Reported in aHUS			Bu et al., 2014
aHUS/C3G	C3	TED	3100T>A	Trp1034Arg	NA	Reported in aHUS			Schramm et al., 2015
AMD	C3	TED	3112G>A	Gly1038Ser	NA				Zhan et al., 2013
aHUS/C3G	C3	TED	3124C>G	Arg1042Gly	0.002	Reported in aHUS			Noris et al., 2010
aHUS/C3G	C3	TED	3124C>T	Arg1042Trp	NA	Reported in aHUS			Schramm et al., 2015
aHUS/C3G	C3	TED	3125G>T	Arg1042Leu	NA	Reported in aHUS			Schramm et al., 2015; Maga et al., 2010
aHUS/C3G	C3	TED	3152A>T	Lys1051Met	0.002	Reported in aHUS			Noris et al., 2010; Schramm et al., 2015
AMD	C3	TED	3180A>T	Arg1060Ser	0.001		Case-control analysis (OR 1.07; p=0.96)		Fritsche et al., 2016; Zhan et al., 2013

AMD	C3	TED	3183A>T	Gln1061His	0.020		Case-control analysis (OR 1.81; p=0.58)	Fritsche et al., 2016; Zhan et al., 2013
aHUS/C3G	C3	TED	3187A>C	Ser1063Arg	0.002	Reported in aHUS		Noris et al., 2010; Schramm et al., 2015
aHUS/C3G	C3	TED	3280G>T	Ala1094Ser	NA	Reported in aHUS		Fremeaux-Bacchi et al., 2013; Schramm et al., 2015
aHUS/C3G	C3	TED	3281C>T	Ala1094Val	NA	Reported in aHUS		Fremeaux-Bacchi et al., 2008; Schramm et al., 2015
aHUS/C3G	C3	TED	3281C>A	Ala1094Asp	NA	Reported in aHUS		Schramm et al., 2015
aHUS/C3G	C3	TED	3284T>G	Ile1095Ser	NA	Reported in aHUS		Fakhouri et al., 2010
Both	C3	TED	3299T>C	Leu1100Pro	0.001	Reported in C3 glomerulopathy	Case-control analysis (OR 0.36; p=0.27)	Fritsche et al., 2016; Zhan et al., 2013
AMD	C3	TED	3305G>A	Gly1102Glu	NA			Zhan et al., 2013
aHUS/C3G	C3	TED	3313A>C	Lys1105Gln	NA	Reported in aHUS		Yoshida et al., 2015
aHUS/C3G	C3	TED	3325C>G	Leu1109Val	NA	Reported in aHUS		Schramm et al., 2015
aHUS/C3G	C3	TED	3341C>T	Pro1114Leu	NA	Reported in aHUS		Bresin et al., 2013; Schramm et al., 2015
aHUS/C3G	C3	TED	3343G>A	Asp1115Asn	NA	Reported in aHUS		Fremeaux-Bacchi et al., 2008; Schramm et al., 2015
AMD	C3	TED	3400C>T	Arg1134Trp	0.003		Case-control analysis (OR 1.33; p=0.71)	Fritsche et al., 2016
AMD	C3	TED	3431C>T	Thr1144Met	0.004		Case-control analysis (OR 2.41; p=0.26)	Fritsche et al., 2016; Zhan et al., 2013
aHUS/C3G	C3	TED	3466G>A	Asp1156Asn	NA	Reported in aHUS		Schramm et al., 2015
aHUS/C3G	C3	TED	3470T>C	Ile1157Thr	NA	Reported in aHUS		Maga et al., 2010; Fan et al., 2013; Martínez-Barricarte et al., 2015
aHUS/C3G	C3	TED	3474C>G	Cys1158Trp	NA	Reported in aHUS		Fremeaux-Bacchi et al., 2008; Schramm et al., 2015
AMD	C3	TED	3475G>A	Glu1159Lys	0.001		Case-control analysis (OR 0.38; p=0.55)	Fritsche et al., 2016; Zhan et al., 2013
aHUS/C3G	C3	TED	3478G>A	Glu1160Lys	NA	Reported in aHUS		Schramm et al., 2015
aHUS/C3G	C3	TED	3481C>A	Gln1161Lys	NA	Reported in aHUS		Fremeaux-Bacchi et al., 2008; Schramm et al., 2015
AMD	C3	TED	3502A>G	Ser1168Gly	0.001			Zhan et al., 2013

Phenotype	Gene	Domain	c.	p.	ExAC (%)	aHUS/C3G comment	AMD comment	Functional Analyses	Source
aHUS/C3G	C3	TED	3625A>G	Lys1209Glu	0.001	Reported in aHUS			Schramm et al., 2015
AMD	C3	TED	3647A>T	Asp1216Val	NA				Zhan et al., 2013
AMD	C3	TED	3655C>T	Arg1219Cys	0.004		Case-control analysis (OR 1.05; p=0.95)		Fritsche et al., 2016; Zhan et al., 2013
AMD	C3	TED	3760C>T	Arg1254Cys	NA		Case-control analysis (OR 0.35; p=0.51)		Fritsche et al., 2016; Zhan et al., 2013
AMD	C3	TED	3811G>T	Ala1271Ser	NA		Case-control analysis (OR 3.28; p=0.46)		Fritsche et al., 2016; Zhan et al., 2013
AMD	C3	TED	3859C>T	Pro1287Ser	NA				Zhan et al., 2013
aHUS/C3G	C3	CUB	3968A>C	Glu1323Ala	NA	Reported in aHUS			Schramm et al., 2015
AMD	C3	MG8	4084G>A	Asp1362Asn	0.005		Case-control analysis (OR 1.29; p=0.73)		Fritsche et al., 2016; Zhan et al., 2013
AMD	C3	MG8	4100T>C	Ile1367Thr	0.080				Zhan et al., 2013
Both	C3	MG8	4148C>A	Thr1383Asn	0.009	Reported in aHUS	Case-control analysis (OR 2.73; p=0.18)		Fritsche et al., 2016; Noris et al., 2010; Schramm et al., 2015
AMD	C3	MG8	4177C>T	Arg1393Trp	0.004		Case-control analysis (OR 0.87; p=0.88)		Fritsche et al., 2016; Zhan et al., 2013
aHUS/C3G	C3	MG8	4294T>C	Tyr1432His	NA	Reported in aHUS			Schramm et al., 2015
AMD	C3	MG8	4319A>C	Asp1440Ala	0.030		Case-control analysis (OR 0.85; p=0.78)		Fritsche et al., 2016; Zhan et al., 2013
AMD	C3	MG8	4364A>G	Glu1455Gly	0.003		Case-control analysis (OR 0.30; p=0.45)		Fritsche et al., 2016; Zhan et al., 2013
AMD	C3	MG8	4369G>C	Asp1457His	0.036		Case-control analysis (OR 2.81; p=0.52)		Fritsche et al., 2016
aHUS/C3G	C3	MG8	4390C>G	His1464Asp	NA	Reported in aHUS			Bresin et al., 2013
AMD	C3	MG8	4441G>A	Ala1481Thr	NA				Zhan et al., 2013
AMD	C3	MG8	4471C>T	Arg1491Trp	0.013		Case-control analysis (OR 0.585; p=0.44)		Fritsche et al., 2016; Zhan et al., 2013

AMD	C3	CTC	4535G>A	Arg1512His	0.014		Case-control analysis (OR 0.484; p=0.47)		Fritsche et al., 2016; Zhan et al., 2013
AMD	C3	CTC	4594C>T	Arg1532Trp	0.007		Case-control analysis (OR 12.29; p=0.04)		Fritsche et al., 2016; Zhan et al., 2013
AMD	C3	CTC	4643G>T	Arg1548Leu	NA				Zhan et al., 2013
Both	C3	CTC	4645C>A	Leu1549Met	0.116	Reported in aHUS	Case-control analysis (OR 1.17; p=0.90)	No influence on FH, MCP, or CR1 binding	Fritsche et al., 2016; Schramm et al., 2015
AMD	C3	CTC	4664C>G	Ser1555Cys	NA		Case-control analysis (OR 0.36; p=0.53)		Fritsche et al., 2016; Zhan et al., 2013
AMD	C3	CTC	4667A>G	Asn1556Ser	0.003		Case-control analysis (OR 0.74; p=0.65)		Fritsche et al., 2016; Zhan et al., 2013
AMD	C3	CTC	4867G>C	Gly1623Arg	NA				Zhan et al., 2013
AMD	C3	CTC	4922A>G	Glu1641Gly	0.003		Case-control analysis (OR 0.74; p=0.68)		Fritsche et al., 2016; Zhan et al., 2013
aHUS/C3G	C3	CTC	4973T>C	Val1658Ala	NA	Reported in aHUS			Sartz et al., 2012
aHUS/C3G	C3	CTC	4985C>T	Pro1662Leu	NA	Reported in aHUS			Ažukaitis et al., 2014

Table S5: Number of unique variants identified in literature.

CFH	aHUS/C3G	AMD	aHUS/C3G and AMD
N-terminus	0	0	0
SCR1	8	6	1
SCR2	7	5	2
SCR3	4	11	1
SCR4	4	6	2
SCR5	0	4	0
SCR6	2	5	2
SCR7	5	3	1
SCR8	5	4	0
SCR9	4	2	0
SCR10	7	6	1
SCR11	6	2	0
SCR12	3	3	1
SCR13	6	1	0
SCR14	8	2	0
SCR15	13	2	0
SCR16	5	3	1
SCR17	7	3	0
SCR18	2	3	0
SCR19	20	0	1
SCR20	32	3	1

CFI	aHUS/C3G	AMD	aHUS/C3G and AMD
N-terminus	0	1	0
FIMAC	4	10	2
SRCR	5	12	1
LA1	2	6	0
LA2	0	4	1
Linkerregion	3	6	1
SP	10	23	13

C3	aHUS/C3G	AMD	aHUS/C3G and AMD
N-terminus	0	0	0
MG1	3	1	0
MG2	2	1	0
MG3	2	5	0
MG4	0	1	1
MG5	2	3	0
MG6a	2	2	0
LNK	1	4	1
ANA	0	4	1
αNT	1	0	1
MG6b	0	0	0
MG7	1	3	0
CUB	0	0	0
TED	22	14	2
MG8	2	8	1
CTC	2	7	1

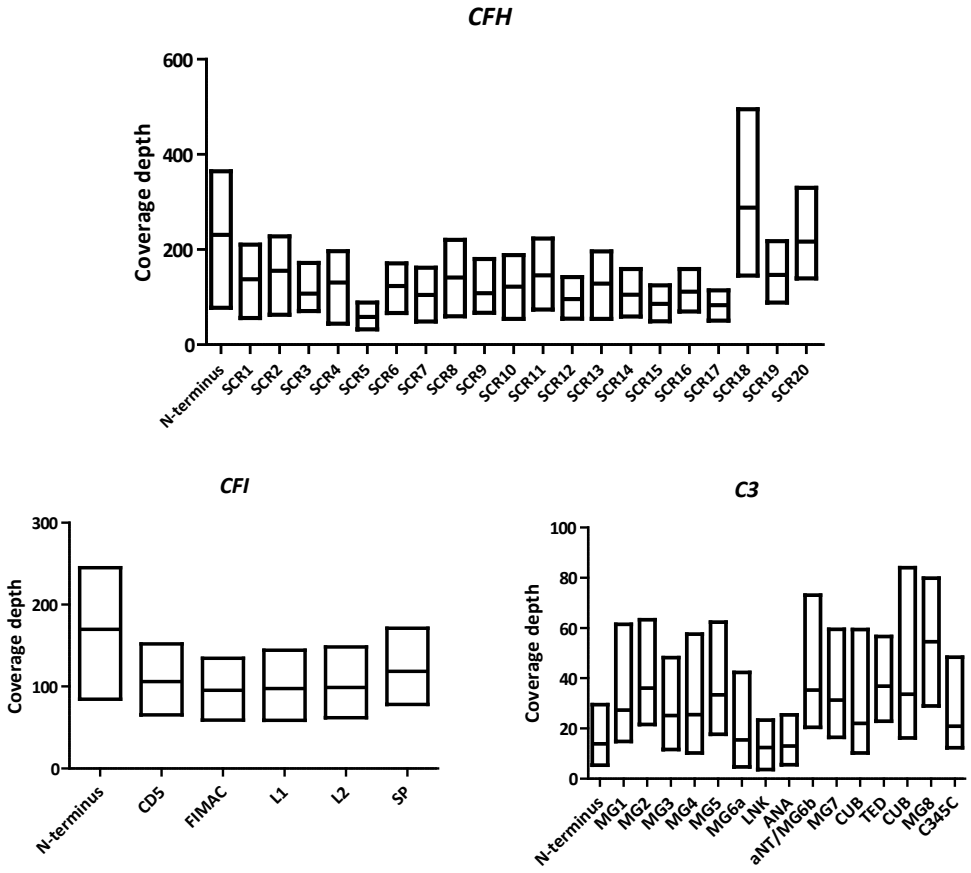

Figure S1: Mean depth of coverage per gene of the AMD cohort. Mean coverage per domain of the AMD cohort with minimum and maximum coverage per complement genes *CFH*, *CFI*, and *C3* in the whole exome sequencing samples.

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Jordi Corominas*
Johanna M. Colijn*
Maartje J. Geerlings
Marc Pauper
Bjorn Bakker
Najaf Amin
Laura Lorés de Motta
Eveline Kersten
Alejandro Garanto
Joost A.M. Verlouw
Jeroen G.J. van Rooij
Robert Kraaij
Paulus T.V.M. de Jong
Albert Hofman
Johannes R. Vingerling
Tina Schick
Sascha Fauser
Eiko K. de Jong
Cornelia M. van Duijn
Carel B. Hoyng
Caroline C.W. Klaver
Anneke I. den Hollander
*these authors contributed equally to this study

Submitted

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WHOLE-EXOME SEQUENCING IN AGE-RELATED MACULAR DEGENERATION IDENTIFIES RARE PROTEIN-ALTERING VARIANTS IN COL8A1, A COMPONENT OF BRUCH'S MEMBRANE

ABSTRACT

Purpose: Genome-wide association studies and targeted sequencing studies of candidate genes have identified common and rare variants that are associated with age-related macular degeneration (AMD). Whole-exome sequencing (WES) studies allow a more comprehensive analysis of rare, coding variants across all genes of the genome, and will contribute to a better understanding of the underlying disease mechanisms. To date, the number of WES studies in AMD case-control cohorts is still scarce and sample sizes are limited. To scrutinize the role of rare protein-altering variants in AMD etiology, we performed the largest WES study in AMD to date, in a large European cohort consisting of 1,125 AMD cases and 1,361 controls.

Design: Genome-wide single-variant and gene-based association analyses of WES data.

Participants: 1,125 AMD cases and 1,361 controls.

Method: rare variants association analysis based on gene-based CMC burden test.

Main outcome measures: Genetic variants associated with AMD.

Results: In this study, we detected a disease burden of rare protein-altering variants in the *COL8A1* gene ($p=7.07x10^{-05}$). The *COL8A1* burden is explained by 14 rare protein-altering variants spread across the protein, which are found more often in cases (22/2,250 alleles, 1.0%) than in controls (11/2,722 alleles, 0.4%). The association of rare variants in the *COL8A1* gene is independent of the common intergenic variant (rs140647181) near the *COL8A1* gene, previously associated with AMD. We demonstrate that COL8A1 localizes at Bruch's membrane, which has a key role in AMD pathogenesis.

Conclusions: This study supports a role for protein-altering variants in the *COL8A1* gene in AMD pathogenesis, and suggests that the previously observed association of the common intergenic variant is driven by effects on *COL8A1*. In this study we for the first time demonstrate the presence of COL8A1 in Bruch's membrane, further supporting the role of *COL8A1* variants in AMD pathogenesis. Protein-altering variants in COL8A1 may alter the integrity of Bruch's membrane, contributing to the accumulation of drusen and the development of AMD.

INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of irreversible vision loss among persons above age 50 years in the developed world.[1,2] The disease is characterized by progressive damage to the retinal pigment epithelium (RPE) and photoreceptors in the macula, ultimately leading to visual impairment and blindness. In the early stages of AMD a spectrum of changes occur, including hypo- and hyperpigmentations of the retina and the formation of extracellular deposits (drusen) in Bruch's membrane.[2] These drusen increase in size and number during the intermediate stages. Two types of AMD can develop in the end stage of the disease. Geographic atrophy (GA), also referred to as the dry form, is characterized by RPE cell atrophy, causing photoreceptor cell death. Choroidal neovascularization (CNV), also called the wet form, is characterized by the formation of new blood vessels, leading to leakage, hemorrhages and sudden loss of vision.

AMD is a multifactorial disease influenced by a variety of environmental factors, including age, smoking history or sunlight exposure during working life.[3,4] There is a large genetic component in AMD etiology, with an estimated heritability between 46% to 71%.[5] Initially, genetic studies in AMD mainly focused on common variants in the population through genome-wide association studies (GWAS) using single nucleotide polymorphism (SNP) microarrays.[6–9] These studies identified genetic variants in or near genes belonging to four main pathways, including the complement system, lipoprotein metabolism, angiogenesis and extracellular matrix remodeling. However, most common genetic variants identified by GWAS are located in non-coding or intergenic regions, and subsequently it is not always apparent which gene near the top-associated SNP is the causative gene.

Involvement of genes in a disease can be further established by identification of functionally relevant variants in the coding regions, which are often rare in the population with a minor allele frequency (MAF) ranging from 0.01% to 1%.8 Thus, several targeted sequencing studies focused on the discovery of rare variants in genes located within AMD loci. In these studies, rare variants were identified in Complement Factor H (*CFH*), Complement Factor I (*CFI*), Complement C3 (*C3*) and Complement C9 (*C9*)[10–14] that are independently associated with AMD. Recently, a GWAS performed by the International AMD Genomics Consortium (IAMDGC) using an exome array enriched with rare variants identified 52 risk-associated variants at 34 genomic loci. Of these 52 variants, seven variants were rare and 45 were common variants.[8]

Testing the association of individual rare variants in single-variant analyses can be challenging, since very large sample sizes are needed to obtain sufficient power.[15] Instead of testing each variant individually, aggregation tests can evaluate the cumulative effects of multiple genetic variants within a gene, leading to an increased study power.[16] Sequence analysis of the

coding regions of 681 genes within AMD-associated loci in 1,676 AMD cases and 745 controls identified a higher burden of rare variants in *CFI* in cases (7.8%) than in controls (2.3%).[12,17,18] Furthermore, evaluation of the cumulative effect of rare protein-altering variants, using exome array data by the IAMDGC, identified a significant burden in four AMD-associated genes: CFH, CFI, Tissue Inhibitor of Metalloproteinases 3 *(TIMP3)* and Solute Carrier Family 16 Member 8 *(SLC16A8)*.[8] A limitation of these studies is that either rare variants in a limited set of genes[12] or a limited number of rare variants across the genome[8] were tested.

Whole-exome sequencing (WES) studies allow a more comprehensive association analysis of coding variants across all genes of the genome with complex traits and diseases.[19] To date, the number of WES studies in AMD case-control cohorts is still scarce and sample sizes are limited. Gene-based analysis using WES of 213 neovascular AMD cases and 1,553 healthy controls from East Asian populations showed association of a variant in Ubiquitin Protein Ligase E3D *(UBE3D)* with AMD.[20] More recently, WES of 39 individuals with bilateral CNV with low genetic risk scores and 36 unaffected controls with high genetic risk, did not det ect any genes that reached genome-wide significance in a gene-based analysis.[21]

The main goal of the present study is the identification of rare protein-altering variants that are associated with AMD. To achieve this goal, we performed WES in a large European cohort consisting of 1,125 cases and 1,361 controls to scrutinize the role of coding variants across the human genome in AMD etiology.

MATERIALS AND METHODS

Study population

A cohort of 2,516 individuals of European ancestry (1,493 females and 1,023 males with a mean age of 79 years) was recruited from the European Genetic Database (EUGENDA, n=799) and the Rotterdam Study (n=1,717).

From EUGENDA (www.eugenda.org), 667 AMD cases and 132 healthy controls were evaluated for this study. Inclusion of individuals took place between December 2005 and June 2014. All participants underwent clinical evaluation by a retinal specialist and were graded for AMD according to the Cologne Image Reading Center (CIRCL) protocol.[22] Fundus photographs and spectral domain optical coherence tomograms were used to classify AMD by the presence of pigmentary changes together with at least 10 small drusen (<63 µm) or the presence of intermediate (63-124 µm) or large drusen (≥125 µm diameter) in the Early Treatment Diabetic Retinopathy Study (ETDRS) grid. Furthermore, late AMD was defined as either AMD with subfoveal geographic atrophy (GA) or choroidal neovascularization (CNV) in at least one eye.

Control individuals were included in the study when they exhibited no signs of AMD in either eye and were at least 65 years of age at inclusion.

The design of the Rotterdam Study has been described previously in detail.[23,24] This prospective, population-based follow-up study that started in 1990 and has follow-up visits every five years. For this analysis, we included a total of 470 AMD cases and 1,247 controls from the Rotterdam Study I sub-cohort aged 55 years and older with WES data. All participants underwent after pupillary dilation, fundus photography of the macula using a 35° film fundus camera (Topcon TRV-50VT; Topcon Optical Company). For the last two follow-up visits a Topcon digital 35° color fundus camera (Topcon TRC 50EX with a Sony DXC-950P digital camera; 0.44 megapixel, Minato, Japan) was used. Fundus photos were graded according to the Rotterdam Classification which is based on the Wisconsin age-related maculopathy grading system[25] (WARMGS) and the modified International Classification System.[26] Cases were participants with early or late AMD, which is at least soft distinct drusen (≥63 μm) in combination with hypo- or hyper-pigmentary changes or soft indistinct drusen (≥ 125 μm) or reticular drusen. Controls were participants above 65 years with no signs of AMD or above 75 years of age with hard or soft distinct drusen (≥63 μm) or pigmentary abnormalities.

In both cohorts, both eyes of all participants were graded separately by experienced graders, who were under the supervision of senior retinal specialists. The worst affected eye was used to classify the individual. Written informed consent was obtained from all participants. The study was approved by the local ethics committees on Research Involving Human Subjects of the participating centers, and all procedures were conducted according to the Declaration of Helsinki principles. The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC and by the Ministry of Health, Welfare and Sport of the Netherlands, implementing the Wet Bevolkingsonderzoek: ERGO (Population Studies Act: Rotterdam Study).

WES capture and variant calling

Genomic DNA of all participants was isolated from blood samples according to standard procedures. DNA was fragmented into 200-400 bp fragments and the exome library was prepared on a Caliper Sciclone NGS workstation (Caliper Life Science, Hopkinton, MA). The exome was captured with the Nimblegen SeqCap EZ Exome v2.0 44Mb kit (Roche Nimblegen, Inc., Madison, WI), covering 329,028 exons and 710 miRNAs. Paired-end sequencing was performed on two Illumina HiSeq2000 sequencer using Illumina TruSeq V3 chemistry (Illumina, Inc., San Diego, CA). High quality reads were mapped to the UCSC hg19 reference genome using the Burrows-Wheeler alignment tool[27]. Variant calling was performed by GATK HaplotypeCaller, following the GATK best practice guidelines [https://software.broadinstitute.org/gatk]. SNVs and indels were filtered separately using GATKs Variant-Quality Score Recalibration (VQSR) module. Variants with a VQSLOD score lower than -7.2 were removed. Variant annotation was done

using ANNOVAR[28] and an in-house pipeline developed by the Department of Human Genetics of the Radboud University Medical Center[29]. Functional effects of variants were predicted by three different prediction algorithms: SIFT[30], PolyPhen-2[31] and CADD[32] (threshold of deleteriousness for CADD ≥ 20). In addition, conservation of candidate variants was estimated by PhyloP (threshold for deleteriousness ≥ 2.7) and Grantham (threshold for deleteriousness ≥ 80).

Data quality control

Stringent quality control steps were performed with PLINK v1.07[33] in order to exclude those positions that had high chances to be false positives. Variants were removed according to the following criteria: i) genotypes with a missing rate higher than 5% of individuals and ii) common variants (MAF>0.05) that were not in Hardy-Weinberg equilibrium (HWE) in controls. After these quality control steps, a total of 744,022 variants were available for analysis. Subject-level quality control was carried out, excluding individuals with a call rate < 95% or an extreme inbreeding coefficient (cutoff ± 0.12).[34] Pairwise identity by descent (IBD) was calculated to confirm the lack of relatedness among all samples (PI-HAT<0.25). A multi-dimensional scaling (MDS) was performed with PLINK v1.07 to obtain the principal components, which were used to confirm that all individuals were clustered as European samples and to correct for population stratification (**Figure S1**; available at www.aaojournal.org). After all quality controls, a cohort of 1,125 AMD cases and 1,361 controls was selected for association analyses.

Statistical analyses

A single variant association test was carried out with RAREMETALWORKER (http://genome.sph.umich.edu/wiki/RAREMETALWORKER) using a linear mixed model. This software performs a score-statistics based rare-variant association analysis, providing single-variant results and a variance-covariance matrix. Linkage disequilibrium (LD) relationships between markers within 1 Mb are stored in the covariance matrix to perform the gene-level analyses. Analysis was performed using an additive model controlling for age, sex, clinic and the first four components. Genome-wide significance levels used for single-variant analysis were defined based on Bonferroni correction (p-value ≤ $5x10^{-08}$).

By definition, single-variant analyses have limited power to detect rare variant associations, especially for limited sample sizes. Association power was increased by evaluating the accumulated association of multiple rare exonic variants within each gene.[35] Gene-based tests were carried out by RAREMETAL[36] using the summary statistics and LD matrices generated in the single-variant analysis. Three different methods were used: the CMC_counts and Variable Thresholds (VT) tests, which are burden tests that assume all alleles to influence the association in the same direction, and the SKAT test, which evaluates risk and protective alleles in order to maximize power. A subset of 308,784 rare protein-altering variants (MAF<0.05) were used in the analysis, to avoid that the major presence of non-protein-altering variants (435,238)

would dilute the disease burden due to deleterious variants. We selected rare variants that alter amino-acid residues (non-synonymous variants), truncate proteins (nonsense and stop-gain variants) or affect RNA splicing (variants affecting the invariate splice donor and splice acceptor sites).

First we focused on the 34 previously reported AMD loci and we applied a Bonferroni-corrected significance threshold based on the 619 genes located within 500 kb of the top-associated SNP in each of the AMD loci (according to [8]) and carrying at least one rare protein-altering variant (p-value < $0.05/619 = 8.07 \times 10^{-05}$). Haploview[37] was used to reconstruct the region of interest to validate that the rare *COL8A1* variants belong to different haplotype blocks than the common risk variant rs140647181 identified in a previous single-variant test[8]. In a secondary analysis, we extended the search of rare variant disease burden to all genes across the genome, applying a Bonferroni-corrected significance threshold of $0.05/17{,}596 = 2.84 \times 10^{-06}$. Quantile-quantile plots of p-values from single-variant analysis and gene-based tests were generated to discard any batch effect or population substructure.

Characterization of phenotypic features of COL8A1 variant carriers

Phenotypic characterization was performed including participants from the Rotterdam Study. AMD features were based on the eye with the most severe phenotype. Glaucoma related features are the mean of both eyes at the last visit during follow-up. Refraction was based on the mean spherical equivalent of both eyes at the last visit during follow-up, or the last visit before cataract extraction. Statistical significance was tested with an independent sample t-test for continuous variables, a chi-squared or Fisher's exact test for dichotomous variables, and a Mann-Whitney U test for drusen area, due to its non-normal distribution. All tests performed were two sided.

Mouse retina staining

Eyes from P60 C57BL/6J wild-type mice were enucleated and embedded in Tissue-Tek® O.C.T. Compound (Sakura Finetek 4583). Seven micrometer sections were dried for 1 h at room temperature. Using the hydrophobic PAP pen (Sigma-Aldrich Z377821-1EA), a circle was drawn surrounding the sections. Retinas were then incubated for 20 min in PBS containing 0.05% Tween (MERCK, 8.22184.0500) and 0.05% Triton X-100 (Sigma Aldrich, 9002-93-1) at room temperature. After blocking in 0.1% ovoalbumin (Applichem, A4344,0250), 0.5% fish gelatine (Sigma-Aldrich, G7041-100G) and 5% bovine serum albumin (Sigma Aldrich, A7906-100G) in PBS for 30 min, primary antibodies were added and incubated overnight at 4°C. Primary antibodies used included rabbit polyclonal anti collagen type VIII alpha 1 (1:50, Sigma-Aldrich, #HPA053107) and rat monoclonal Laminin beta-1 (1:50, ThermoFisher Scientific, MA5-14657). Retinas were washed 4 x 5 min in PBS, incubated with the goat anti-rabbit Alexa 568 (1:500, Life technologies, A11006) and goat anti-rat Alexa 488 secondary antibody (1:500, Life technologies,

A11006) for 45 min at room temperature (dilution 1:500 in blocking solution). Nuclei staining with 4',6-diamidino-2-phenylindole (1:8000, ITK, #0100-20) was combined with the secondary antibody incubation. Sections were then washed 4 x 5 min in PBS, rinsed in MilliQ-purified water and mounted in Prolong Gold anti-fade reagent (Life technologies, P36930). Imaging was performed using a Zeiss Z1 Imager. All images were taken at the same intensity. An image with ZEN software was created to obtain TIFF or JPEG files.

RESULTS

Whole exome sequencing

We performed WES on 2,516 unrelated individuals (1,125 cases and 1,361 controls), obtaining an average of 2,8 billion bases per individual and a mean coverage of 63X. After variant calling and recalibration, a total of 759,450 variants were identified, being 754,503 single nucleotide variants (SNVs) and 4,947 insertions or deletions (indels). Of the complete set of variants, 7.6% (57,571) were common variants and the remaining 92.4% (701,879) were classified as rare variants with a MAF <0.05. Genotype data obtained from WES were checked for concordance with the genotype data of a customized Illumina exome array,[8] available for a subset of the study population (n=1,330). Variants genotyped by both WES and exome array (n=80,779) had a concordance rate of >99%, demonstrating the high quality of our sequencing data and high accuracy of our genotype calling.

Single variant and gene-based association analyses

We first performed a genome-wide single-variant analysis using the WES data of 1,125 AMD cases and 1,361 controls of European ancestry. Common variants were included in order to identify the association signals obtained from these variants. Results confirmed association of variants in the *CFH* and *Age-Related Maculopathy Susceptibility 2* (*ARMS2)* genes with AMD in this cohort.[8] Two common coding variants in *CFH* (rs1061170 [$p = 4.24x10^{-11}$] and rs1061147 [$p = 3.30x10^{-10}$]) and one common variant in *ARMS2* (rs10490924 [$p = 1.89x10^{-09}$]) were associated with AMD above the threshold of genome-wide significance (p-value ≤ $5x10^{-08}$; see **Figure S2 and Figure S3** available at www.aaojournal.org).

Subsequently, we evaluated the burden of rare protein-altering variants in genes at previously identified AMD loci using gene-based burden tests. For this analysis 619 genes were selected that are within 500 kb of the top-associated SNP at 34 AMD loci identified in a recent GWAS[8] (**Table S1**; available at www.aaojournal.org). A CMC burden test (applying genomic control λ = 0.940) showed a significant disease burden in the *COL8A1* gene ($p = 7.07x10^{-05}$) (**Figure 1**).

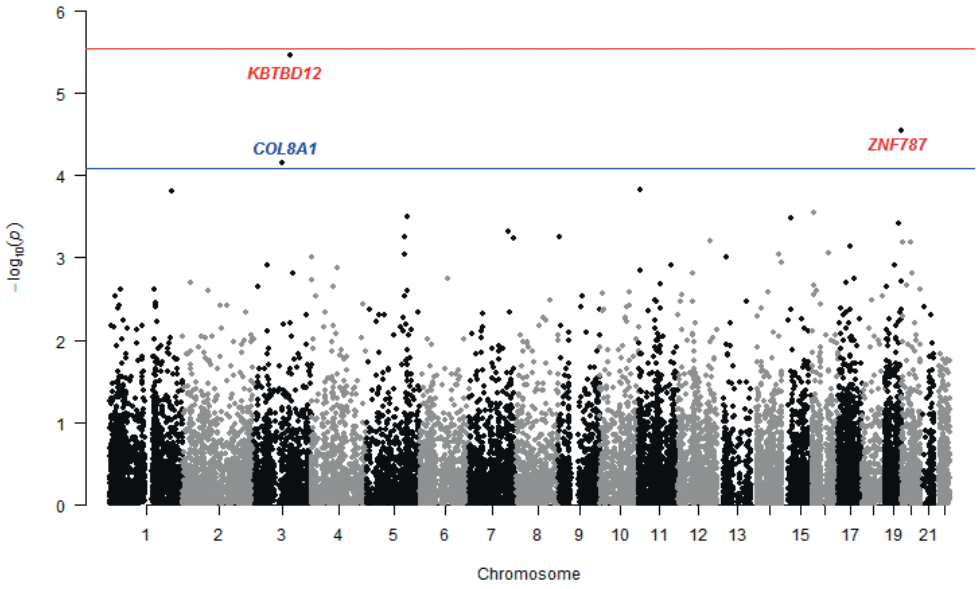

Figure 1. Manhattan plot of gene-based analysis for disease burden, testing protein-altering variants in 18,215 genes using the CMC test. The blue line indicates the significance threshold (p-value < 0.05/619 = 8.07×10^{-05}) for testing 619 genes located in or near AMD-associated loci. The *COL8A1* gene reaches the significance threshold, and is depicted in blue. The red line indicates the genome-wide significant threshold (p-value < 0.05/17,596 = 2.84×10^{-06}) for genes outside the AMD-associated loci. The *KBTBD12* and *ZNF787* genes do not reach genome-wide significance, and are depicted in red. Bonferroni correction was applied to both significance thresholds.

We then expanded the burden analysis to protein-altering variants across the genome. The CMC burden test (applying genomic control $\lambda = 1.057$) showed a suggestive association in the *KBTBD12* ($p = 3.50 \times 10^{-06}$) and *ZNF787* genes ($p = 2.89 \times 10^{-05}$), but these associations did not reach the genome-wide significance level (Figure 1). The signal at the *KBTBD12* gene did reach the genome-wide significance threshold when the SKAT test was applied ($p = 4.45 \times 10^{-07}$). In both tests, the association signal was mainly driven by the effect of one rare variant (rs148151101; p-value = 1.52×10^{-06}). However, this particular variant in *KBTBD12* was not associated with AMD in an exome array analysis in a cohort of 16,144 AMD cases and 17,832 controls of European ancestry by the IAMDGC (p = 0.387).[8]

Rare variant burden in the COL8A1 gene

We next determined whether the rare variant burden in *COL8A1* is independent of the previously identified AMD-associated common variant (rs140647181) near the *COL8A1* gene[8]. This common variant is intergenic, located 560 kb downstream of *DCBLD2* and 177 kb upstream of *COL8A1*. To evaluate the independence between both signals, we reconstructed the haplotype blocks at the *COL8A1* locus. Several recombination events between the rare protein-altering

variants in the *COL8A1* gene and rs140647181 were observed (**Figure 2**). These results support that the rare variant burden observed in this study is independent of the common intergenic variant previously associated with AMD.

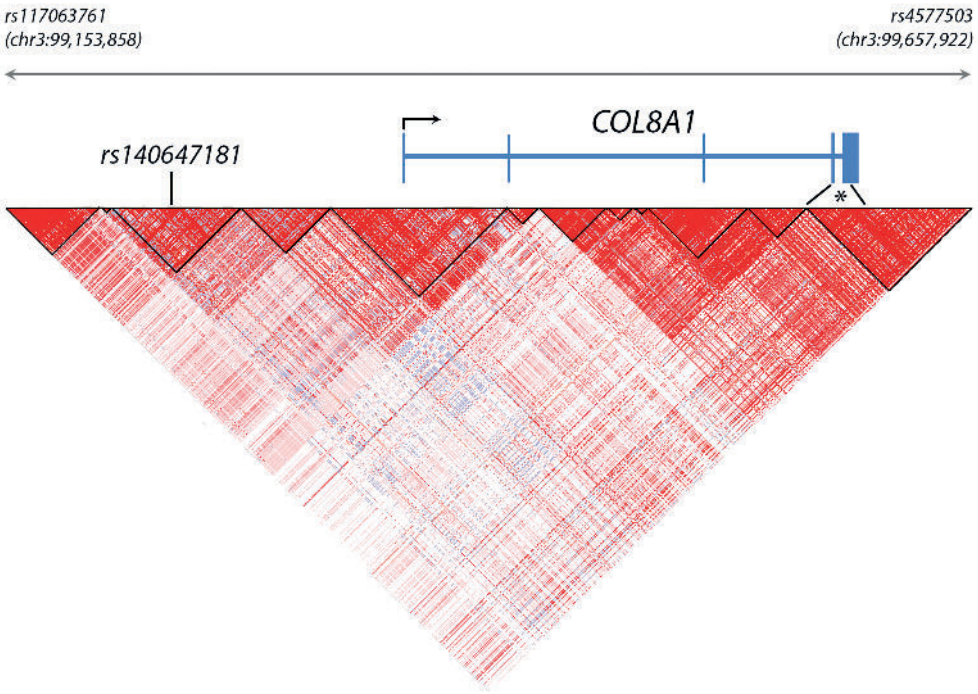

Figure 2. Haploblock structure of the genomic region encompassing the COL8A1 gene and the AMD-associated SNP rs140647181 located 177 kb upstream of COL8A1 (chr3:99,153,858-99,657,922). A Haploview plot was generated based on common SNVs extracted from the 1000 Genomes Phase 3 dataset. In black are depicted the most likely haploblocks in this region. This haplotype block distribution shows that coding variants identified in the *COL8A1* gene (indicated with an asterisk, *) are not located in the same haplotype block as rs140647181.

Table 1. Rare protein-altering variants identified in the *COL8A1* gene in 1,125 AMD cases and 1,361 controls.

Protein change	cDNA change	Domain	PhyloP*	Grantham*	Sift (score)*	Polyphen2 (score)*	CADD*	Counts cases (n=2,250)	Counts Controls (n=2,722)	Single variant p-value	Single variant OR (95% CI)	Burden test p-value	Burden test OR (95% CI)
V58A	173T>C	NC2	4.317	64	Damaging (0.014)	Pos. damaging (0.646)	19.7	0	1	0.56	0.78232 (0.34-1.79)	$7.07x10^{-05}$	1.34 (1.16-1.55)
M70T	209T>C	NC2	4.216	81	Damaging (0.004)	Benign (0.001)	22.5	1	0	0.11	1.9552 (0.85-4.47)		
A96V	287C>T	NC2	1.266	64	Tolerated (1)	Benign (0)	5.6	1	0	0.06	2.18674 (0.96-5)		
P193R	578C>G	COL1	4.028	103	Damaging (0.02)	Pos. damaging (0.463)	22.1	0	1	0.61	0.80691 (0.35-1.84)		
R225Q	674G>A	COL1	6.782	43	Tolerated (0.328)	Benign (0.008)	22.9	1	1	0.48	1.23339 (0.69-2.21)		
A250V	749C>T	COL1	2.257	64	Tolerated (0.338)	Benign (0)	2.5	1	1	0.83	0.93868 (0.52-1.68)		
R362Q	1085G>A	COL1	3.041	43	Tolerated (0.105)	Benign (0.071)	20.5	6	3	0.06	1.3026 (0.99-1.72)		
G414*	1240G>T	COL1	9.803	NA	NA	NA	39	2	0	0.01	2.14633 (1.20-3.85)		
E520K	1558G>A	COL1	9.828	56	Tolerated (0.296)	Pos. damaging (0.945)	21.6	1	0	0.82	1.1015 (0.48-2.52)		
H668Q	2004C>G	NC1	2.728	24	Damaging (0.004)	Pro. damaging (0.989)	25.9	0	1	0.45	0.72856 (0.32-1.66)		
G695D	2084G>A	NC1	9.873	94	Damaging (0.005)	Pro. damaging (0.977)	26.7	4	2	0.08	1.3489 (0.96-1.89)		
G711E	2132G>A	NC1	9.873	98	Damaging (0.014)	Pro. damaging (1)	26.7	1	0	0.08	2.1159 (0.92-4.84)		
L741F	2223G>T	NC1	0.615	22	Tolerated (0.084)	Benign (0.366)	22.2	2	1	0.11	1.47754 (0.92-2.38)		
M744I	2232G>C	NC1	9.477	10	Tolerated (0.186)	Benign (0.001)	24.8	2	0	0.13	1.56374 (0.87-2.81)		

* Thresholds for deleteriousness: PhyloP ≥ 2.7; Grantham ≥ 80; Sift ≤ 0.1; ‡ Polyphen ≥ 0.4; CADD ≥ 20. Pro. = Probably; Pos. = Possibly.

The *COL8A1* burden is explained by 14 rare protein-altering variants spread across the protein (**Figure 3**), which are found more often in cases (22/2,250 alleles, 1.0%) than in controls (11/2,722 alleles, 0.4%) (**Table 1**). Six variants, including one nonsense variant (p.G414*) and 5 missense variants (p.M70T, p.A96V, p.E520K, p.G711E, p.M744I), were identified only in cases but not in controls, and 5 additional variants (p.R225Q, p.A250V, p.R362Q, p.G695D, p.L741F) were found at a higher frequency in cases than in controls. The nonsense variant p.G414* is predicted to lead to a premature termination in the COL1 domain, or may cause nonsense-mediated decay of the *COL8A1* mRNA. Two missense variants (p.G695D and p.G711E) are predicted to be deleterious with all conservation and pathogenicity tests used, and have a CADD score ≥20, which classifies them among the top 0.75% most deleterious mutations that are found in the human genome (**Table 1**). The two missense variants p.G695D and p.G711E affect two highly conserved amino acid residues in the non-collagenous 1 (NC1) domain (**Figure 3**).

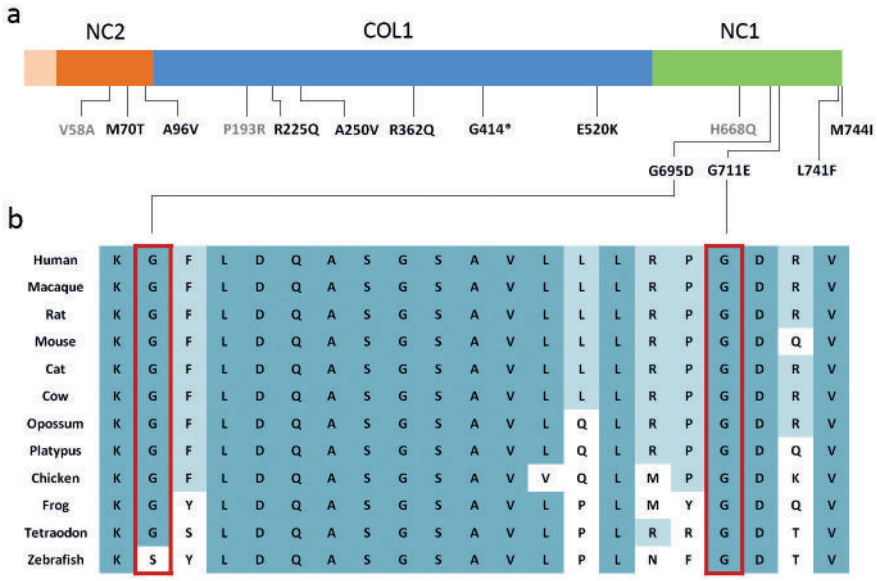

Figure 3. Location and conservation of protein-coding variants in COL8A1. (A) Location of rare protein-altering variants identified in AMD cases and controls in the different COL8A1 domains: triple-helical region (COL1), non-collagenous domain 1 (NC1) and 2 (NC2). Variants detected only in control individuals are depicted in gray. (B) Alignment of COL8A1 protein sequences of different species. Boxed missense variants identified in AMD patients, predicted to be deleterious in all conservation and pathogenicity tests (Table 1), affect highly conserved glycine residues in the NC1 domain.

Table 2. Comparison of phenotypic features between carriers and non-carriers of *COL8A1* variants.

Protein change	***COL8A1* variant and AMD** (N=16)	***COL8A1* variant, no AMD** (N=11)	**No *COL8A1* variant and AMD** (N= 450)
Age at last visit	79.6 (SD 6.3)	82.5 (SD 7.9)	80.0 (SD 6.5)
Spherical Equivalent	-0.37 (SD 1.86)*	1.14 (SD 1.83)	1.26 (SD 2.29)*
Mild myopia (-3 to -6D, %)	3/16 (19%)	1/11 (9%)	23/437 (5%)
Severe myopia (<= -6D, %)	0/16 (0%)	0/11 (0%)	2/437 (0%)
Corneal curvature, mm	7.72 (SD 0.32)	7.58 (SD 0.26)	7.70 (SD 0.26)
IOP, mmHg	13.8 (SD 3.0)	14.3 (SD 2.8)	13.9 (SD 3.3)
VCDR	0.36 (SD 0.18)	0.37 (SD 0.24)	0.32 (SD 0.18)
Subtype of AMD, number	3 GA, 0 CNV, 0 Mixed, 13 Early	-	29 GA, 21 CNV, 21 Mixed, 379 Early
Drusen area in grid, median	2% (range 0-37%)	0% (range 0-2%)	2% (range 0-75%)
Presence of hyperpigmentation, %	14/16 (88%)	1/11 (9%)	287/450 (64%)
Presence of reticular drusen, %	0/16 (0%)	0/11 (0%)	27/450 (6%)
Presence of drusen outside grid, %	10/16 (63%)	7/11 (64%)	not available

* p=0.005 independent samples t-test (2-tailed) between AMD cases carrying a *COL8A1* variant (N=16) and AMD cases without variants in *COL8A1* (N=437), *t*-value=2.81 degrees of freedom=451. IOP: intraocular pressure. VCDR: vertical cup-disc ratio. GA: geographic atrophy (dry AMD). CNV: choroidal neovascularization (wet AMD). Mixed AMD: GA and CNV.

Phenotypic features of COL8A1 variant carriers

We examined the effect of the *COL8A1* variants on the AMD phenotype in participants from the Rotterdam Study only, because it is a population-based cohort study without prior selection on phenotype. This group consists of 16 AMD cases carrying a *COL8A1* variant, 11 individuals carrying a *COL8A1* variant without AMD, and 450 AMD cases without a *COL8A1* variant (**Table 2**). Features of early AMD were not significantly different between *COL8A1* carriers and non-carriers with AMD, although *COL8A1* carriers had a somewhat higher proportion of hyper-pigmentary changes (p = 0.062). No statistically significant differences were found for glaucoma-related features such as intraocular pressure (IOP) and vertical cup-disc ratio (VCDR). The groups differed significantly in spherical equivalent; *COL8A1* carriers being more myopic (p=0.005). However, there was no significant difference in the proportion of mild and severe myopia.

Localization of COL8A1 to Bruch's membrane

Localization of COL8A1 in the retina has not yet been described in the literature. In order to assess whether COL8A1 is localized at Bruch's membrane, the main AMD disease site, we performed immunohistochemistry on retinas of wild-type C57BL/6J adult mice. Laminin Beta-1 (LAMB1) was used as a marker for Bruch's membrane.[38] The co-immunostaining of LAMB1 and COL8A1 robustly demonstrated that both proteins localize at Bruch's membrane (**Figure 4**). In addition, COL8A1 showed some expression in the photoreceptor layer, being most evident at the outer plexiform layer (OPL), the synaptic region between the photoreceptor cells and the inner nuclear layer cells. To exclude that the staining was due to background staining derived from the use of secondary antibodies, we performed the same procedure without adding primary antibody (**Figure S4**; available at www.aaojournal.org). This confirmed that the COL8A1 and LAMB1 staining observed at Bruch's membrane is due to the primary antibody.

DISCUSSION

In this study we aimed to scrutinize the role of rare protein-altering variants in AMD etiology using WES. By focusing on rare and functionally relevant variants in the coding regions, we sought to determine the causality of genes in the disease. Since most top SNPs identified in GWAS studies for AMD are in non-coding or intergenic regions,[8] it is not always apparent which gene near the top-associated SNP is the causative gene. In this study, WES analysis in 1,125 AMD patients and 1,361 controls revealed a significant burden of rare protein-altering variants in the *COL8A1* gene in AMD. The *COL8A1* burden is explained by 14 rare protein-altering variants spread across the protein, which are found more often in cases (22/2,250 alleles, 1.0%) than in controls (11/2,722 alleles, 0.4%). The association of rare variants in the *COL8A1* gene is independent of the common AMD-associated intergenic variant rs140647181, located 560 kb downstream of *DCBLD2* and 177 kb upstream of *COL8A1*.[8] No rare-variant burden was observed in the *DCBLD2* gene, nor in other genes at the same AMD locus. Taken together, these findings support that the previously observed association of the common intergenic variant rs140647181 is driven by effects on *COL8A1* rather than other genes at the locus.

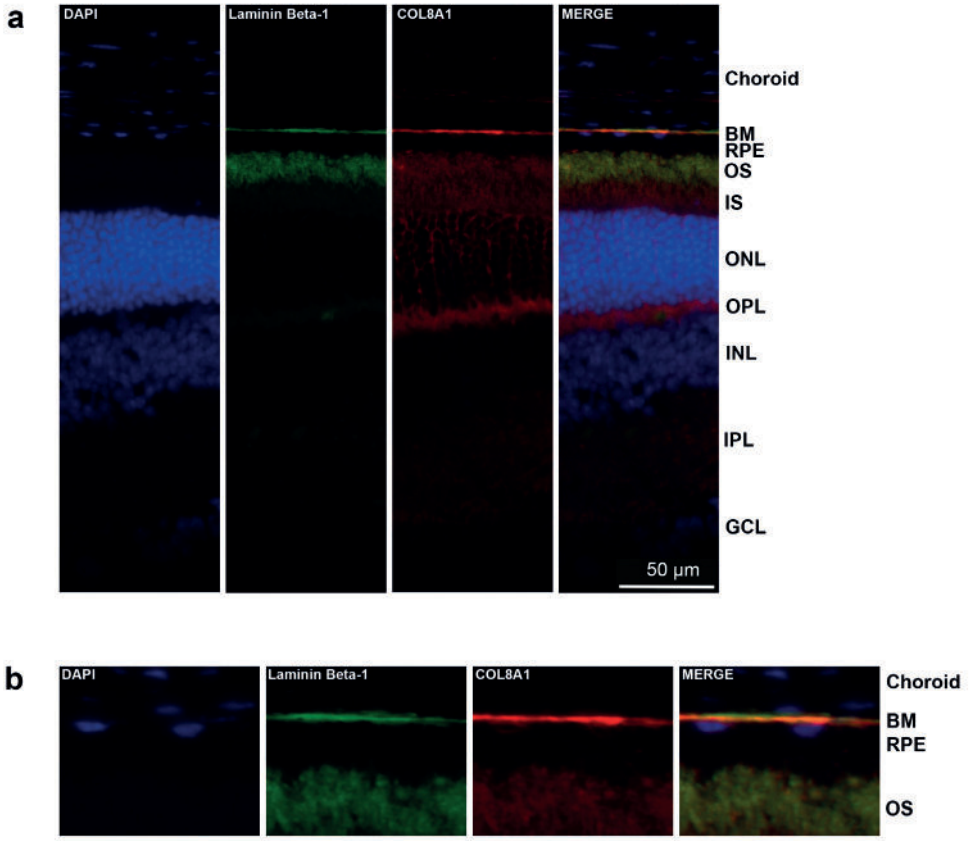

Figure 4. Immunodetection of COL8A1 in mouse retinas. (A) The localization of COL8A1 (in red) was studied on P90 retinas derived from wild-type C57BL/6J mice. Laminin Beta-1 (LAMB1, in green) was used as a Bruch's membrane marker. COL8A1 co-localizes with LAMB1 at Bruch's membrane. COL8A1 staining also showed a weaker signal in other layers of the retina. (B) Magnifications of the outer region of the retina, where the co-localization between LAMB1 and COL8A1 can be appreciated. DAPI (in blue) was used to stain cell nuclei. BM: Bruch's membrane; RPE: retinal pigment epithelium; OS: outer segments; IS: inner segments; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer and GCL: ganglion cell layer.

COL8A1 encodes one of the two alpha chains of collagen type VIII, which is a major component of ocular basement membranes.[39] Several studies have investigated the association between alterations in genes encoding the two subunits of collagen VIII (COL8A1 and COL8A2) and ocular abnormalities such as myopic choroidal neovascularization, anterior segment dysgenesis and thin corneal stroma.[40–43] Although several studies postulated a role for COL8A1 in ocular basement membranes, so far no published data confirmed the localization of COL8A1 in Bruch's membrane. There are several lines of evidence to support that Bruch's membrane

plays a crucial role in AMD. Due to its location, Bruch's membrane is intensively involved in the exchange of numerous biomolecules, nutrients and waste products between the RPE and the choroidal capillary bed.[44] A disturbed integrity or stability of Bruch's membrane can lead to accumulation of these products in drusen, or can weaken the physical barrier against the invasion of new blood vessels into the retina.[45] In this study, we demonstrate for the first time the presence of COL8A1 in Bruch's membrane, further supporting the role of *COL8A1* variants in AMD pathogenesis.[46]

Protein-altering variants in COL8A1 may lead to structural alterations in Bruch's membrane, which can be responsible for the development of AMD.[41] Interestingly, we describe 14 rare protein-altering variants in COL8A1, including one nonsense variant (p.G414*), and two deleterious missense variants (p.G695D and p.G711E) that affect highly conserved residues in the C-terminal NC1 domain. The NC1 domain mediates proper folding of the protein and the assembly of collagen VIII and X into polygonal lattices.[47–49] Therefore, these COL8A1 variants may lead to an aberrantly folded protein, impairing transport of the protein to Bruch's membrane, or altering Bruch's membrane integrity or stability. Consequently, this may contribute to the development of early AMD. In our study, we observe a higher proportion of hyper-pigmentary changes in AMD patients carrying *COL8A1* variants, albeit non-significant. Larger patient populations are needed to validate this finding. Previous studies have implicated COL8A1 in retinal angiogenesis by mediating proliferation and migration of endothelial cells,[41] suggesting that *COL8A1* variants could contribute to the development of neovascularization in late AMD. Interestingly, *COL8A1* variants seem to contribute to refractive error, although the contribution to severe myopic errors was insignificant.

The findings described here need to be interpreted in light of several strengths and limitations. We demonstrated that WES with relatively large cohorts is an efficient strategy to detect burden in AMD-associated genes. Previous studies that detected disease burden in AMD were focused on predefined gene-sets using targeted sequencing[12] or predefined variant-sets using exome arrays,[8] while our study performed a comprehensive exome-wide search for rare variants using WES. The main advantage of performing WES is that it enables the identification of all rare variants present in coding regions across the genome, allowing a more comprehensive evaluation of the disease burden than other approaches based on a limited set of genes or variants. An AMD disease burden has been previously described in *CFH, CFI, TIMP3* and *SLC16A8,*[8,12] but these findings were not confirmed in our study. The reason may lie in the fact that despite having a relatively large cohort, our study may not have sufficient power to detect these associations. In the study by Fritsche et al., a larger cohort was used consisting of 16,144 AMD cases and 17,832 controls.[8] However, the majority of the *COL8A1* variants (11/14) identified by WES in our study were not present on the exome array that was used by Fritsche et al., which may explain why a *COL8A1* disease burden was not observed in that study.[8] In

addition, differences in study designs and populations, case definition, geographical origin, statistical tests used or correction for confounding factors may explain the different results observed among these studies.

In conclusion, we performed the largest exome-wide sequence analysis of rare protein-altering variants in AMD to date, and detected a disease burden in the *COL8A1* gene. A common intergenic variant near this gene was previously associated with AMD risk.[7,8] but no protein-altering variants within the gene have been described in AMD so far. This work supports a role for protein-altering variants in *COL8A1* gene in AMD pathogenesis, and suggests that the previously observed association of the common intergenic variant is driven by effects on *COL8A1*. In this study we for the first time demonstrate the presence of COL8A1 in Bruch's membrane, further supporting the role of *COL8A1* variants in AMD pathogenesis. Protein-altering variants in COL8A1 may alter the integrity of Bruch's membrane, contributing to the accumulation of drusen and the development of AMD. This study shows that WES provides a fruitful approach for gene and variant identification in complex disorders, such as AMD. Collaborative efforts among the scientific community are needed to perform even larger exome- or genome-wide sequencing studies,[50] which will further increase our understanding of the genetic architecture and disease mechanisms of AMD.

SUPPLEMENTARY INFORMATION

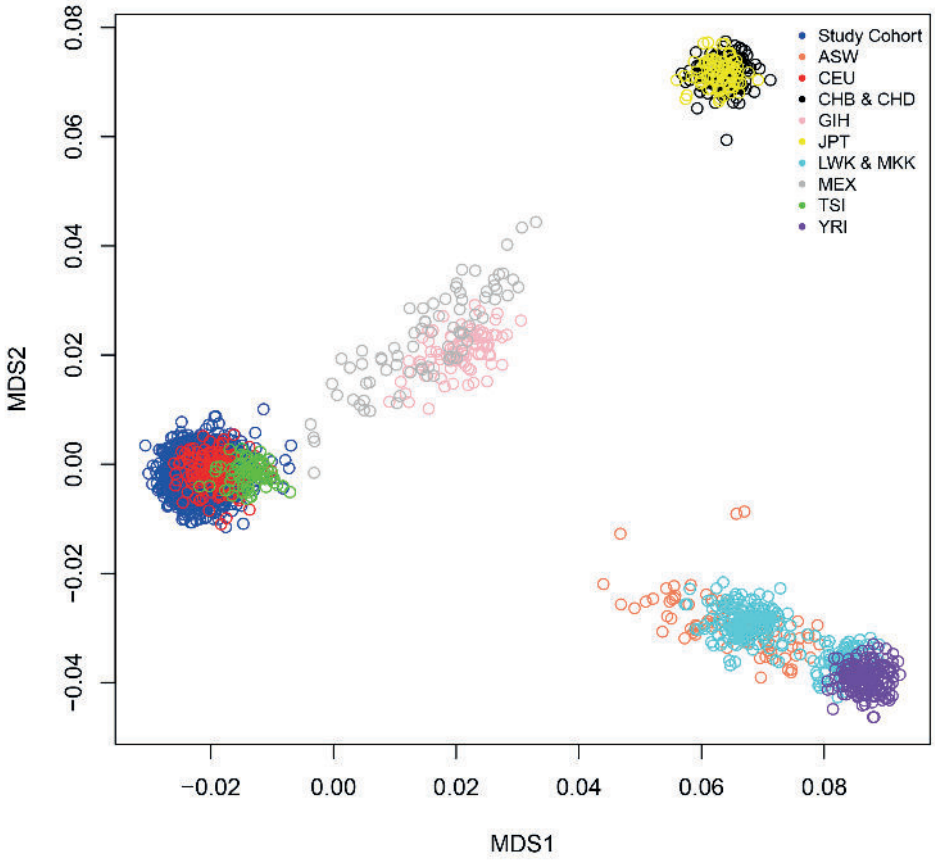

Figure S1. Multidimensional scaling analysis. The pair-wise genetic distances was used to identify relationships between our study cohort and the HapMap populations. The first two components were plotted in this figure.

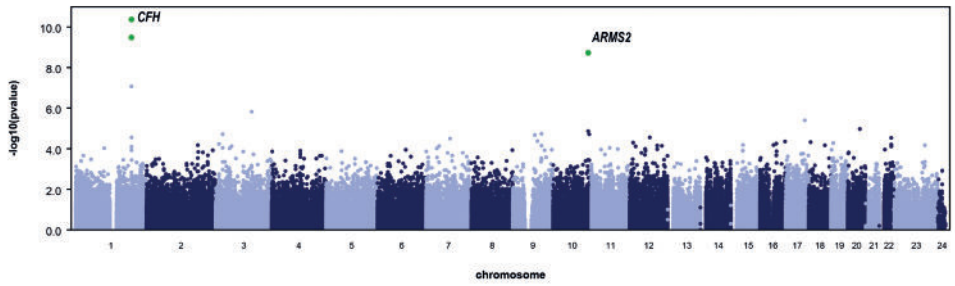

Figure S2. Manhattan plot for single-variant analysis. The association results of each variant (depicted in dots) are plotted against the genomic position. Significant variants after Bonferroni correction are depicted in green.

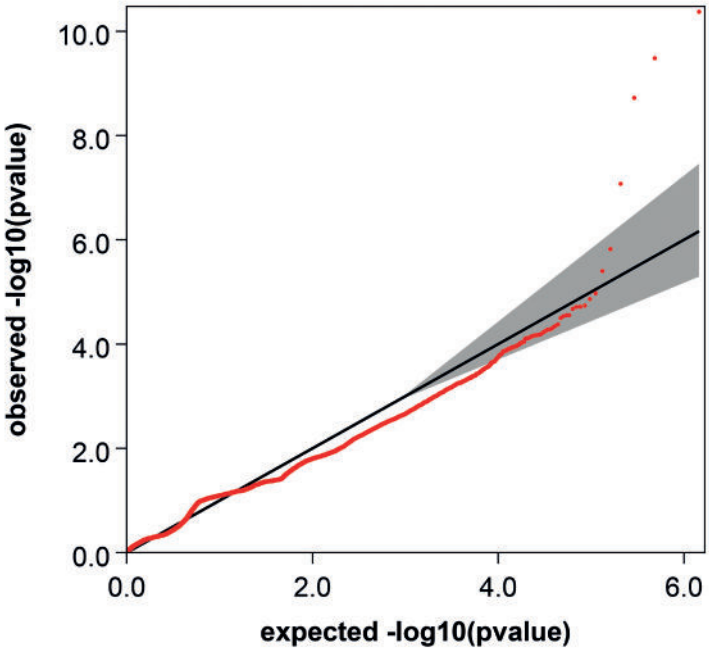

Figure S3. QQ-plot for single-variant analysis. The observed p-values [-log10(p-value)] from the single-variant association analysis for all variants (red dots). The identity (lack of association) is depicted with a black line, together with the 95% confidence interval (grey shadow). The observed p-values are corrected by genomic control using of 0.990.

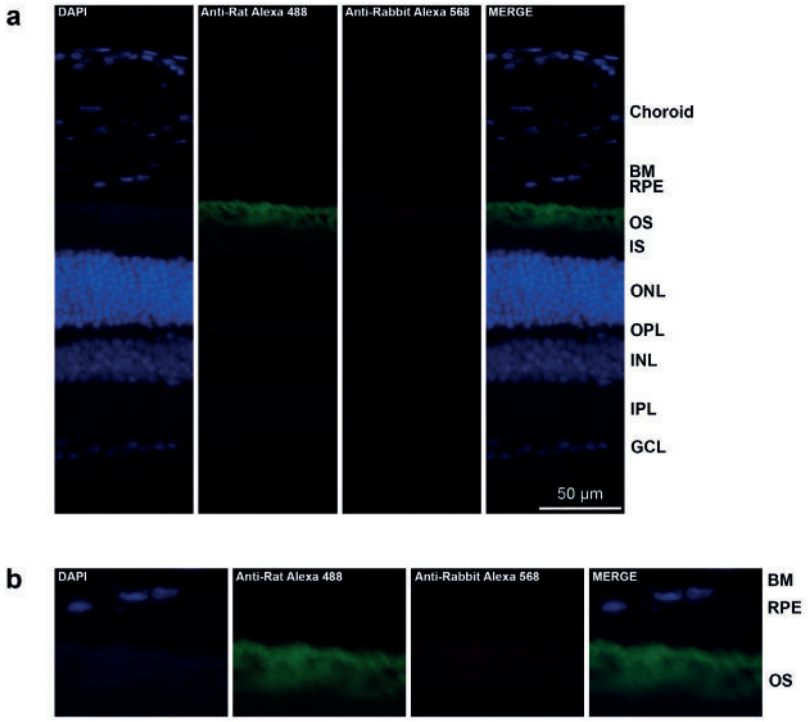

Figure S4. Assessment of the background signal derived from the secondary antibodies. (A) Assessment of the goat anti-rabbit Alexa 568 (in red) and goat anti-rat Alexa 488 (in green) staining on P90 retinas derived from wild-type C57BL/6J mice. No signal was detected in the red channel (secondary antibody used to detect COL8A1). However, some signal in the outer segment of the photoreceptors was detected in the green channel (secondary antibody against LAMB1), but not at the Bruch's membrane. Therefore, the signal at the outer segment is due to the secondary antibodies and therefore it was considered background. (B) Magnifications of the region of interest. DAPI (in blue) was used to stain the nuclei of the different cells. BM: Bruch's membrane; RPE: retinal pigment epithelium; OS: outer segment; IS: inner segment; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer and GCL: ganglion cell layer.

Table S1. List of 619 genes carrying rare protein-altering variants that are located within AMD-associated regions.

Gene ID	Chr	Start bp	End bp	Strand
KCNT2	1	196194909	196578355	-1
CFH	1	196621008	196716634	1
CFHR3	1	196743925	196763203	1
CFHR1	1	196788887	196801319	1
CFHR2	1	196788898	196928356	1
CFHR4	1	196819371	196888102	1
CFHR5	1	196946667	196978804	1
F13B	1	197008321	197036397	-1
ASPM	1	197053258	197115824	-1
ZBTB41	1	197122810	197169672	-1
CRB1	1	197170592	197447585	1
DENND1B	1	197473878	197744826	-1
IRS1	2	227596033	227664475	-1
RHBDD1	2	227700297	227863931	1
COL4A4	2	227867427	228028829	-1
COL4A3	2	228029281	228179508	1
MFF	2	228189867	228222550	1
TM4SF20	2	228226872	228246711	-1
AGFG1	2	228336868	228425930	1
C2orf83	2	228474806	228498036	-1
SLC19A3	2	228549926	228582728	-1
PRICKLE2	3	64079543	64431152	-1
ADAMTS9	3	64501333	64673676	-1
DCBLD2	3	98514785	98620533	-1
COL8A1	3	99357319	99518070	1
FILIP1L	3	99548985	99833357	-1
TBC1D23	3	99979844	100044095	1
NIT2	3	100053545	100075710	1
TOMM70A	3	100082275	100120242	-1
LNP1	3	100120037	100175163	1
TMEM45A	3	100211463	100296288	1
COL25A1	4	109731877	110223813	-1
SEC24B	4	110354928	110462052	1
CCDC109B	4	110481361	110609874	1
CASP6	4	110609785	110624739	-1
PLA2G12A	4	110631145	110651233	-1
CFI	4	110661852	110723335	-1
GAR1	4	110736666	110745893	1
RRH	4	110749150	110765760	1
LRIT3	4	110769358	110793471	1
EGF	4	110834040	110933422	1
ELOVL6	4	110967002	111120355	-1
RAI14	5	34656342	34832732	1
TTC23L	5	34838938	34899561	1
RAD1	5	34905369	34919094	-1
BRIX1	5	34915481	34926101	1
DNAJC21	5	34929698	34959069	1
AGXT2	5	34998206	35048198	-1
PRLR	5	35048861	35230794	-1
SPEF2	5	35617946	35814713	1
IL7R	5	35852797	35879705	1
CAPSL	5	35904397	35938881	-1
UGT3A1	5	35951112	36001130	-1
UGT3A2	5	36035119	36071460	-1
LMBRD2	5	36098514	36152063	-1
SKP2	5	36152091	36184421	1
RANBP3L	5	36248536	36302216	-1
OSMR	5	38845960	38945698	1
RICTOR	5	38938021	39074510	-1
FYB	5	39105338	39274630	-1
C9	5	39284364	39424970	-1
DAB2	5	39371780	39462402	-1
ZNF318	6	43274872	43337216	-1
ABCC10	6	43395104	43418168	1
DLK2	6	43418090	43424370	-1
TJAP1	6	43445261	43474294	1
LRRC73	6	43474707	43478424	-1
POLR1C	6	43477440	43497323	1
YIPF3	6	43479565	43484728	-1
XPO5	6	43490072	43543812	-1
POLH	6	43543887	43586701	1
GTPBP2	6	43573053	43596899	-1
MAD2L1BP	6	43597277	43608689	1
RSPH9	6	43612783	43640336	1
MRPS18A	6	43639040	43655528	-1
VEGFA	6	43737921	43754224	1
C6orf223	6	43968317	43973695	1
MRPL14	6	44081194	44095194	-1
TMEM63B	6	44094651	44123256	1
CAPN11	6	44126548	44152139	1
SLC29A1	6	44187242	44201888	1
HSP90AB1	6	44214824	44221620	1
SLC35B2	6	44221833	44225291	-1
NFKBIE	6	44225903	44233500	-1
TMEM151B	6	44238203	44275243	1
TCTE1	6	44246480	44265458	-1
AARS2	6	44267391	44281063	-1
SPATS1	6	44310397	44344904	1
CYP3A43	7	99425636	99463718	1
OR2AE1	7	99473610	99474680	-1
TRIM4	7	99474581	99517223	-1
GJC3	7	99520892	99527243	-1
AZGP1	7	99564343	99573780	-1
ZKSCAN1	7	99613204	99639312	1
ZSCAN21	7	99647390	99662661	1

ZNF3	7	99661656	99680171	-1
COPS6	7	99686577	99689823	1
MCM7	7	99690351	99699563	-1
AP4M1	7	99699172	99707968	1
TAF6	7	99704693	99717464	-1
CNPY4	7	99717236	99723134	1
MBLAC1	7	99724317	99726118	1
C7orf43	7	99752043	99756338	-1
GAL3ST4	7	99756867	99766373	-1
GPC2	7	99767229	99774995	-1
STAG3	7	99775186	99819111	1
GATS	7	99798276	99869837	-1
PVRIG	7	99815864	99819113	1
SPDYE3	7	99905325	99919819	1
PILRB	7	99933737	99965356	1
PILRA	7	99965153	99997719	1
ZCWPW1	7	99998449	100026615	-1
MEPCE	7	100026413	100031741	1
PPP1R35	7	100032905	100034188	-1
C7orf61	7	100054238	100061894	-1
TSC22D4	7	100060982	100076902	-1
NYAP1	7	100081550	100092422	1
AGFG2	7	100136834	100165842	1
LRCH4	7	100169855	100183776	-1
SAP25	7	100169855	100171270	-1
FBXO24	7	100181605	100198740	1
PCOLCE	7	100199800	100205798	1
MOSPD3	7	100209725	100213007	1
TFR2	7	100218039	100240402	-1
ACTL6B	7	100240720	100254084	-1
GNB2	7	100271154	100276797	1
GIGYF1	7	100277130	100287071	-1
POP7	7	100303676	100305118	1
EPO	7	100318423	100321323	1
ZAN	7	100331249	100395419	1
EPHB4	7	100400187	100425121	-1
SLC12A9	7	100424442	100464631	1
TRIP6	7	100464760	100471076	1
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SLC35C2	20	44978167	44993043	-1
ELMO2	20	44994688	45061704	-1
ZNF334	20	45129701	45142198	-1
CTCFL	20	56071035	56100708	-1
PCK1	20	56136136	56141513	1
ZBP1	20	56178902	56195632	-1
PMEPA1	20	56223448	56286592	-1
C20orf85	20	56725960	56736206	1
ANKRD60	20	56793551	56803709	-1
RAB22A	20	56884752	56942563	1
VAPB	20	56964178	57026157	1
APCDD1L	20	57034157	57090187	-1
C22orf42	22	32544993	32555309	-1
RFPL2	22	32586422	32600718	-1
SLC5A4	22	32614465	32651328	-1
RFPL3	22	32750872	32757148	1
BPIFC	22	32809834	32860471	-1
FBXO7	22	32870663	32894818	1
SYN3	22	32908539	33454358	-1
TIMP3	22	33197687	33259030	1
ELFN2	22	37764000	37823505	-1
MFNG	22	37865101	37882439	-1
CARD10	22	37886400	37915549	-1
CDC42EP1	22	37956454	37965412	1
LGALS2	22	37966255	37978623	-1
GGA1	22	38004481	38029571	1
SH3BP1	22	38030661	38062939	1
PDXP	22	38054734	38062941	1
LGALS1	22	38071615	38075813	1
NOL12	22	38077680	38170137	1
TRIOBP	22	38093011	38172563	1
H1F0	22	38201114	38203442	1
GCAT	22	38203912	38213183	1
GALR3	22	38219389	38221502	1
ANKRD54	22	38226862	38245334	-1
EIF3L	22	38244875	38285414	1
MICALL1	22	38301664	38338829	1
C22orf23	22	38339528	38349676	-1
POLR2F	22	38348614	38437922	1
SOX10	22	38366693	38383429	-1
PICK1	22	38452318	38471708	1
SLC16A8	22	38474141	38480100	-1
BAIAP2L2	22	38480896	38506677	-1
PLA2G6	22	38507502	38601697	-1
MAFF	22	38597889	38612518	1
TMEM184B	22	38615298	38669040	-1
CSNK1E	22	38686697	38794527	-1
KCNJ4	22	38822332	38851205	-1

KDELR3	22	38864067	38879452	1
DDX17	22	38879445	38903665	-1
DMC1	22	38914954	38966291	-1
FAM227A	22	38974125	39052634	-1

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10

GENERAL DISCUSSION

The general aim of this thesis was to elucidate the role of rare genetic variants in the pathogenesis of age-related macular degeneration (AMD). We set out to identify novel genetic causes of AMD, and to understand the effect of identified rare genetic variants on the disease mechanisms. The following section elaborates on the primary findings of this thesis, places these findings in a broader perspective, and discusses the clinical and scientific relevance.

RARE VERSUS COMMON GENETIC VARIANTS

Studying rare genetic variants in complex traits has advantages and challenges when compared to studying common genetic variants.[1,2] First, studying rare variants requires different study designs compared to common variants. In addition, rare variants seem to contribute only a small proportion of the disease risk of complex disease compared to common variants. On the other hand, the effect of common variants is usually subtle and not always apparent, while rare coding variants may result in clear functional consequences. Moreover, rare variants can have very large effect sizes on the disease risk opposed to common variants, which usually have relatively small effects. Finally, the frequency of rare variants can considerably vary among populations.

Rare variant study designs

Implicating rare genetic variants in disease pathogenesis requires immense sample sizes or specific study designs. Examples of study designs that can be utilized to find rare variant associations include: sampling extreme phenotypes, using population isolates or sub-populations, performing targeted sequencing or studying families.[3,4]

Extreme phenotype sampling is applicable in quantitative traits and focuses on extreme presentations at the tails of the phenotypic distribution. While this technique may boost the statistical power, it is sensitive to sampling bias and outcomes may be difficult to reflect on the general population.[4,5] A recent study[6] used extreme phenotype groups, based on a cumulative risk score, to identify associations outside the regions known to be associated with risk for AMD.

The use of population isolates in rare variant association studies is based on the concept that they have a reduced genetic diversity. Disease-causing rare variants may therefore be found at higher frequencies within these groups compared to the general population.[4] For example, sequencing of Amish individuals identified a population-specific, risk-conferring variant in *CFH*.[7]

A study design performing targeted sequencing of genes or pathways of interest allows for a reduction in time and costs. A reduced gene set provides increased accuracy, allows larger samples sizes to be analyzed and requires a smaller DNA input.[8] Targeted sequencing of candidate genes led to the identification of rare genetic variants in *C3* and *CFI*.[9-11]

A study design using families relies on the occurrence of disease-causing variants at high frequency in affected family members compared to unaffected individuals, or affected families compared to the general population.[4] In chapter 2 and 3 we used families with multiple individuals affected by AMD to identify rare genetic variants, which we replicated in a case-control cohort.

Contribution of common versus rare variants to complex disease

Common variants are estimated to be the major contributor to disease risk in complex disorders. Park and colleagues[12] showed that variants involved in complex diseases are skewed towards MAF >20%, rather than low frequency (MAF <5%) variants.[12] It has been suggested that rare variants (MAF <1%) can explain a portion of the disease heritability in complex diseases.

Genetic studies performed to date in AMD suggest that common variants have a much larger contribution to disease risk than low frequency variants. The 52 independently associated variants reported by the International AMD Genomics Consortium explain 27.2% of the disease variability, which included a 1.4% contribution from seven rare variants. Additional large studies using targeted sequencing, exome sequencing or whole genome sequencing may expand our knowledge on the contribution of rare variants to AMD pathogenesis. The samples size required to properly determine the impact of rare variants on disease risk was estimated at over 25,000 patients.[13]

Functional effect of common versus rare variants

Common variants in complex disorders are commonly identified through genome-wide association studies (GWAS). The index (tag) SNP tags multiple SNPs within the same haplotype block, and is in high linkage disequilibrium (LD) with those other SNPs. The index SNPs is often non-coding and the functional effect is not always apparant.[14] Moreover, due to the high linkage disequilibrium of the index SNPs with other SNPs in the same haplotype block, the index SNP does not necessarily need to be the causative variant. Determining the functional effect of common variants on the disease mechanisms can therefore be challenging.

On the other hand, rare coding variants may result in clear functional consequences, critically changing the amino acid, or leading to a premature stop in the protein. An example of a rare variant with a clear functional effect is *CFH* p.Arg1210Cys. This variant is one of the strongest rare genetic risk factors in AMD.[9,13] Human serum albumin binds to the mutant cysteine,

interfering with FH's ability to bind with ligands and abolishing all other FH functional domains. As a result the variant results in a partial FH deficiency.[15,16]

Effect sizes of common versus rare variants

In complex diseases, an inverse relation exists between the odds ratio and the frequency of a genetic variant in the population (**Figure 1**).[12] Variants like *CFH* rs570618 (in high linkage disequilibrium with *CFH* p.Tyr402His), *ARMS2* rs3750846 (in high linkage disequilibrium with *ARMS2* p.Ala69Ser), and protective *CFH* variants rs10922109, are unique examples of common variants with relatively large effect sizes (OR>2, or in the case of protective variant OR<0.5). These variants plot in the lower right corner of **Figure 1** and are effectively identified by GWAS.

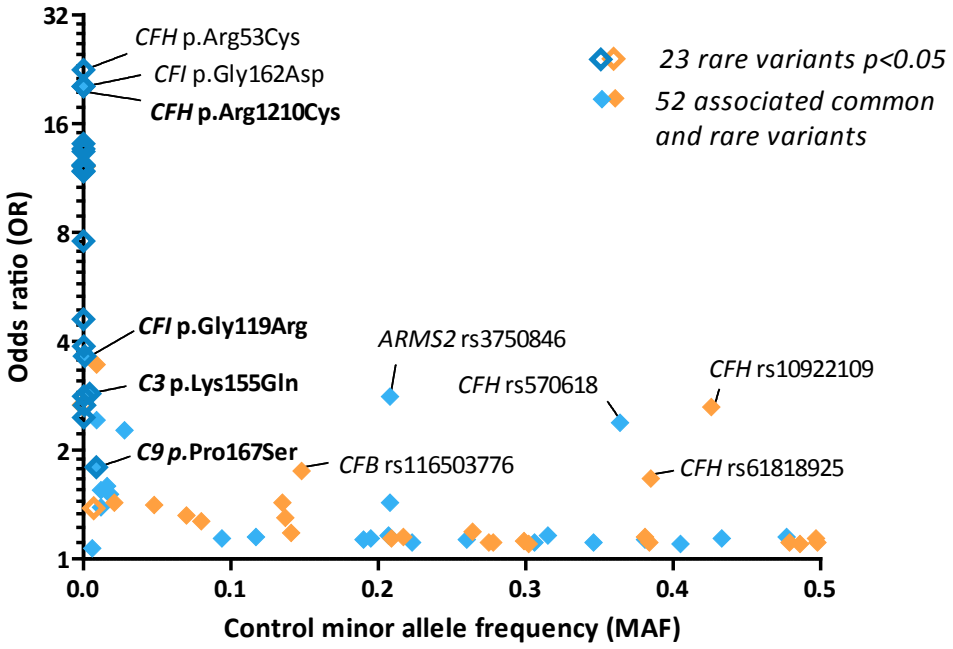

Figure 1: Frequency and effect-size of AMD associated variants. Odds ratio (OR; y-axis) and minor allele frequency (MAF; x-axis) with MAF based on control individuals without AMD. The filled diamonds represent 52 independently associated rare and common variants found in 34 loci as published by Fritsche et al.,[13] (supplementary table 4). The outlined diamonds represent 23 rare variants in *CFH*, *CFI*, *C3*, and *C9* that reached P<0.05 as reviewed in Chapter 1B[17] (supplementary table 1). Variants *CFH p.*Arg1210Cys, *CFI* p.Gly119Arg, *C9* p.Pro167Ser and *C3* p.Lys155Gln (in bold) are shown both as filled and outlined diamonds. Protective ORs are transformed (1/OR) and depicted in orange.

Rare variants with small effect sizes plot in the left lower corner of **Figure 1**. These type of variants are difficult to detect and require large sample sizes to reach genome-wide significance. Rare variants with a large effect size, like *CFH* p.Arg1210Cys and *CFH* p.Arg53Cys, (upper left corner of **Figure 1**), behave like highly penetrant Mendelian mutations, and are often found clustering in subpopulations or families. A challenge when studying rare variants, however, is that the effect size can only be estimated if the variant is identified in a sufficient amount of cases and controls to calculate the odds ratio. For rare variants identified in only a small number of cases or families, the effect size cannot accurately be calculated, making it difficult to provide an accurate risk assessment to carriers of such rare variants.

Distribution of rare variants among populations

Allele frequencies can exhibit large diversity among populations. While most common variants are shared worldwide, rare variants have the tendency to cluster in specific populations or families. For example, the p.Arg1210Cys variant in *CFH* was first reported in a case-control study from the United States[18]. While some studies could replicate the finding[9,13,15], other Caucasian studies [11,19-21] and Asian studies [22,23] were unable to replicate its strong association. Such population-specific rare variants in particular tend to have a strong functional effect.[24] In **chapter 5** we describe the distribution of risk-associated rare genetic variants identified by the International AMD Genomic Consortium.[13] We observed that the frequency of two of the variants (*CFH* rs121913059 and rs35292876 [p.Arg1210Cys]) show a different distribution among populations. However, all variants in the study had comparable risk estimates across the geographical regions.

The occurrence of population-specific variations has implications for genetic counseling and carrier screening in both diagnostic and research settings. For example, carriers of specific rare genetic variants in the complement genes that increase complement activation may benefit more from complement inhibiting therapy than those who do not carry such variants[25]. Personalized treatment aiming at complement-activating rare variants in clinical trials may only be applicable to specific populations where these variants are sufficiently common.

FAMILY STUDIES TO IDENTIFY RARE GENETIC VARIANTS IN AMD

In this thesis we used family studies to identify rare genetic variants associated with AMD. A challenge that we encountered when studying rare variants in families, is that rare variants do not segregate with the disease. We observed an earlier age at symptom onset, and higher prevalence of a positive family history in individuals carrying rare genetic variants. Recommendations are provided on how family studies can be performed to identify additional rare variants.

Rare genetic variants identified in families with AMD

Family studies have shown a higher prevalence of AMD characteristics among relatives of AMD patients than in control families.[26-30] The genetic risk of families with multiple affected individuals can largely be explained by a clustering of common risk variants in these families.[27] In families, for which common variants cannot explain the high burden of disease, the heritability is thought to lie within rare variants with large effect sizes.[31] These rare variants can be difficult to detect in case-control studies due to their rare frequency, appearance in only a few families, or occurrence in specific populations.[7,32,33] Recently, a number of studies using WES have successfully identified novel rare genetic variants by analyzing multiple affected individuals of large AMD families (**Table 1**).[7,25,32-37]

We observed rare variants *CFI* p.Gly119Arg, *C9* p.Pro167Ser and *C3* p.Lys155Gln, which were previously implicated in AMD pathogenesis,[9-11,20] in five of 22 families affected by AMD (**chapter 2** and **3**). Although these variants aggregated within these families, they did not segregate completely with the disease phenotype (**Chapter 2**). Our observations show that rare variants found in large case-control studies are also observed in AMD families, but that these variants are not necessarily inherited in a dominant Mendelian inheritance pattern. Additional factors, like common variants and environmental factors may explain why one individual develops AMD while his/her siblings carrying the same rare variants do not. In a case-control setting, carriers of *CFI* p.Gly119Arg, *C9* p.Pro167Ser and *C3* p.Lys155Gln did show an earlier age at symptom onset. Furthermore, individuals carrying one of these rare variants reported a positive family history for AMD more often than patients with AMD who did not.

In **chapter 3** we screened the same 22 families for additional rare variants residing in complement genes *CFH, CFI C3*, and *C9*. We identified six new rare variants, namely *CFH* p.Ser193Leu, *CFH* p.Arg175Gln, *CFI* p.Pro553Ser, *CFI* p.Leu131Arg, *C9* p.Arg118Trp, and *C3* p.Arg161Trp. These rare variants were found more often in affected than unaffected individuals, both in the families and the case-control cohort, but again the variants did not show perfect segregation with the disease phenotype in families affected by AMD.

Table 1: Rare genetic variants identified in AMD families using whole exome sequencing.

Study	Family structure and segregation	Gene	Variant(s)	Detection method	Additional genotyping	Functional assessment
Ratnapriya et al.,[34] 2014	Two generations; five affected and two unaffected; complete segregation.	Fibrillin 2 (*FBN2*)	c.3430G>A; p.Glu1144Lys	Whole-exome and Sanger sequencing and TaqMan Assay.	Sequencing of exon 24-34 in 196 individuals identified four additional rare variants in *FBN2*. Common variant c.2893G>A; p.Val965Ile in *FBN2* showed a suggestive association (OR 1.10; p=3.79x10-5) in a case-control study of 11511 individuals.	FBN2 was identified in human RPE and choroid but not in retina by RNA-seq and immunoblot. Staining of human eyes detected FBN2 in BM, choroid and sclera. FBN2 staining was reduced in aged and AMD eyes.
Hoffman et al.,[7] 2014	One generation; four affected and two unaffected; incomplete segregation.	*CFH*	c.1507C>G; p.Pro503Ala	Whole-exome sequencing and custom array.	Genotyped 973 Amish individuals, including 95 self-reported AMD cases and 2371 non-Amish individuals. An additional 15 *CFH* p.Pro503Ala carriers were identified in the Amish cohort (8 affected and 5 unaffected). No carriers were identified in the non-Amish.	-
Yu et al., [33] 2014	Two families: A) one generation of five affected and one unaffected. B) two generations; eleven affected and one unaffected. Complete segregation in both families.	*CFH*	A) c.269A>G; p.Asp90Glu B) c.157C>T; p.Arg53Cys	Whole-exome sequencing, Taqman assay and custom array.	Screening of 2421 individuals revealed four *CFH* Asp90Glu carriers affected by AMD, but *CFH* Arg53Cys was not found.	Serum FH levels were normal in 22 variant carriers, with normal protein secretion. Both variants show reduced co-factor activity for FI and severely impaired DAA. *CFH* Arg53Cys shows reduced binding affinity.

Pras et al., [32] 2015	Three two generation families; A) two affected and four unaffected; B and C) five affected and two unaffected; complete segregation.	*CFI* in family A and B. Hemicentin (*HMCN1*) in family C.	*CFI* c.1234G>A; p.Val412Met *HMCN1* c.4162delC; p.Pro1388Hisfs*14	Whole-exome and Sanger sequencing and restriction digest assay.	For *CFI*, whole-exome sequencing data of 146 individuals revealed two *CFI* carriers. Restriction digest assay revealed 10 carriers in 200 unrelated population controls (Jewish Tunisian). For *HMCN1*, the variant was not identified in the whole-exome sequencing cohort and was unknown in public databases.	-
Duvvari et al., [35] 2016	Five families; at least two affected individuals; incomplete segregation.	Screening 289 candidate genes.	A total of 9 variants were detected in 4 families. In two families no variants remained.	Whole-exome sequencing.	In addition to the 14 family members, 12 sporadic cases were screened for the candidate gene list using WES.	-
Saksens et al.,[37] 2016	22 families; at least three affected individuals; incomplete segregation.	Screening for specific rare variants	*CFI* c.355G>A p.Gly119Arg; *C9* c.499C>T; p.Pro167Ser, and *C3* c.463A>C; p.Lys155Gln	Whole-exome and Sanger sequencing, KASPAR assay.	In addition to the 174 family members, 2975 individuals were screened for four rare genetic variants	Systemic complement activation (C3d/C3 ratio) was associated with AMD status but not with carrying one of the rare variants.
Wagner et al., [36] 2016	Four families: A) one generation with five affected and two unaffected. B and C) two affected siblings and D) two generations with three affected and one unaffected.	*CFH*	A) c. c.575G>T; p.Cys192Phe B) c.790+1G>A C) c.524G>C; p.Arg175Pro D) c.380G>A; p.Arg127His	Whole-exome sequencing.	-	Carriers had lower serum antigenic FH levels compared to noncarrier family members. For the variants identified in families A to C, the mean serum levels were reduced compared to 45 noncarrier unaffected controls.

Geerlings et al., [25] 2017	22 families; at least three affected individuals; incomplete segregation.	Screening candidate genes *CFH*, *CFI*, *C3*, and *C9*.	*CFH c.*578C>T; p.Ser193Leu, *CFH* c.524G>A; p.Arg175Gln, *CFI c.*1657C>T; p.Pro553Ser, *CFI* c.392T>G; p.Leu131Arg, *C9* c. 352 C>T; p.Arg118Trp, and *C3* c.481 C>T; p.Arg161Trp	Whole-exome and Sanger sequencing, KASPAR assay.	In addition to the 174 family members, 3198 individuals were screened for specific rare variants in , *CFH*, *CFI*, *C3*, and *C9*.	FI serum levels of *CFI* Gly119Arg and *CFI* Leu131Arg carriers was reduced and C9 serum level i*n C9* Pro167Ser carriers was elevated compared to noncarriers. Carriers of *CFH* (Arg175Gln and Ser193Leu) and *CFI* (Gly119Arg and Leu131Arg) variants have an impaired ability to degrade C3b.

Abbreviations: BM = Bruch's Membrane; DAA = decay-accelerating activity

Considerations for unbiased approaches to identify rare variants in AMD families

In **chapters 2** and **3** we focused on rare variants in genes that were previously associated with AMD. As a next step, the families could be used to identify rare variants in genes not yet known to be associated with AMD, using unbiased approaches. An approach that could be used is to search for variants that are shared among siblings. However, siblings naturally share a great number of genetic variants. Searching for variants shared among the affected siblings in an unbiased fashion will therefore result in large list of overlapping genetic variants.[4,38]

Inclusion of one or more unaffected siblings can drastically narrow down the number of candidate variants by excluding variants shared with the unaffected sibling(s). However, it is of great importance that the unaffected individual is of similar age, or preferable older, than its affected siblings due to the large contribution of age on disease onset. If the unaffected individuals within a family affected by AMD are younger, chances are the individual will still develop AMD, and he/she can therefore not be considered a proper control. Increasing the number of affected and unaffected individuals in the analysis will reduce the probability of co-segregation by chance.[38] The families used in **chapters 2** and **3** are densely affected by AMD, meaning that at least three siblings were diagnosed with AMD.

A more powerful approach would be the use of multiple generation families, however, for age-related diseases obtaining such families is difficult. Moreover, since rare genetic variants do not necessarily segregate with the disease in the family, filtering for variants shared among affected siblings, and not present in unaffected siblings, may unintentionally remove disease-associated variants.

Testing for rare variants associations in AMD families

Rather than searching for variants that segregate in families, association designs can be used to identify rare disease-associated variants in families. Families are more genetically homogeneous compared to the general population. Therefore, an increased occurrence of disease-associated rare variants can be expected in affected family members.

Using burden tests or variance component tests on a family dataset may successfully detect genetic associations. Numerous rare variant association methods for family-based samples have been published, each designed with a specific inheritance pattern and study design in mind.[46] For example, some analyses are restricted to quantitative traits[47-49] instead of dichotomous (binary) traits like AMD. Others strictly perform linkage analyses without further association analyses,[50-52] or require parent-child information.[39,48] Furthermore, assumptions such as complex or monogenic inheritance and common or rare disease frequency should be taken into account. Examples of rare variant based tests for complex disorders are provided in **Table 2**.

Table 2: Rare variant based tests for complex and dichotomous traits in families.

Methods	Test	Study design	Source
RV-TDT: Rare variant transmission disequilibrium	Transmission disequilibrium test (TDT)	Parent-child trio; Requires affected and unaffected individuals	He et al.,[39] 2014
GEE-KM: generalized estimating equations-based kernel association	Burden, C-alpha	Requires a large dataset of sib-pairs; prospective data can be included	Wang et al.,[40] 2013
PedCMC: pedigree Combined Multivariate and Collapsing	Burden	Protective and deleterious variants are collapsed together	Zhu & Xiong,[41] 2012
PedGene: gene-level kernel and burden association tests with disease status for pedigree data	Burden, C-alpha	Requires affected and unaffected individuals	Schaid et al.,[42] 2013
FARVAT: family-based rare variant association test	Burden, C-alpha, SKAT-O	Introduction of SKAT-O allows analysis of rare variants with different directions	Choi et al.,[43] 2014
Rare-variant association test based on identical by decent (IBD) sharing	Burden and variance-component	Uses alleles shared identical by descent nd requires a large dataset of sib-pairs	Epstein et al.,[44] 2015
mFARVAT: multivariate family-based rare variant association tool	Burden, SKAT-O	Can include both homogeneous and heterogeneous approaches	Wang et al.,[45] 2016

Wang and colleagues[46] compared a number of rare variant based tests for dichotomous traits, and reported FARVAT and PedGene to be the most efficient methods when examining families. Ultimately, FARVAT was recommended as it was most computational efficient and could incorporate SKAT-O statistics. However, these results were obtained using extended families consisting of 10 individuals and across three generations.[43] In case of smaller families, like our dataset, one should combine both family and case-control data,[53] add weighted common and rare genetic variants, and combine it with the SKAT-O algorithm.

WHOLE EXOME SEQUENCING AND IMPLICATIONS OF FINDING 'A RARE VARIANT'

In this thesis we used whole-exome sequencing (WES) to detect rare genetic variants in AMD patients and control individuals for **all chapters**, except chapter 5. In rare variant studies, the far majority of variants detected by sequencing are rare or seen in only one individual.[54,55] It can be difficult to assess if these rare variants are harmful or benign based on association with the

disease alone. There are numerous approaches at different experimental levels to assess the pathogenicity of rare variants. In this section a number of strategies on assessing the disease causality of rare variants are discussed.

Exome quality and annotation

Correct interpretation of data starts with a high quality dataset. Quality of the dataset should be guarded in the data analysis pipeline and subsequent quality control steps. Data analysis consists of base calling, read alignment, variant calling and annotation.[56] Base calling is the initial step in which the nucleotides are identified within a single read. The quality of the calling is dependent on sample quality and if samples are uncontaminated. During read alignment the short DNA strands (50-400 base pairs) are compared to a reference as a puzzle. The larger the size of the strands, the more reliably can they be aligned to the reference. The problem is that some parts of the genome are highly repetitive or similar.[57] The exome enrichment kit (Nimblegen SeqCap EZ Exome v2.0 44Mb kit) used for the studies in this thesis, captures 60 to 90 basepairs per probe, which could lead to mismapping or low coverage in repetitive regions. Sanger sequencing can be used to validate if the variants are correctly called.

Furthermore, exome kits may vary in the number of genes that are effectively captured, number of reads mapped back to the reference genome, number of duplicate 'pair' reads, and ability to capture variations.[57] Quality control checks the number of variants per individual, fraction of variant sites and the ratio of transition and transversion. Variant calling quality depends on the depth of coverage across the exome, for which the recommended minimum is 20X at 80-95% of the exome.[58] For **chapter 2**, **3**, and **6** we confirmed all identified WES variants by Sanger sequencing. In **chapter 4**, **7** and **8** we used a minimum number of (variant) reads, and for **chapter 9** we report a mean coverage of 63X. During the last step, annotation, and functional effects of variants are added by tools like ANNOVAR.[59]

Variant interpretation

The power of rare variant analyses can be improved if the functional variants could be separated from the those without a functional effect. One way of predicting the functional effect of a variant is through *'in silico'* prediction software (**Table 3**).

These algorithms, based on conservation, structural information or amino acid physic-chemical properties, estimate the likelihood of variant to be harmful. For the *CFI* gene these prediction algorithms seem well equipped. A correlation is seen between the prediction score (PolyPhen2) and Factor I (FI) serum levels. Variants predicted to be pathogenic generally have low FI levels, and these low serum FI levels strongly increase the risk of developing advanced AMD.[67] Unfortunately, these algorithms are not always reliable, as can been seen for two genetic variants that are strongly associated with AMD. *CFH* p.Arg1210Cys and *C3* p.Lys155Gln

are variants that are both predicted to be tolerated and benign by SIFT[60] and PolyPhen[61]. Functional assays, however, show that *CFH* p.Arg1210Cys results in a partial FH deficiency, and *C3* p.Lys155Gln results in reduced binding to FH and cleavage of C3b.[9,10,15,18,20]

Table 3: Most used software to predict the effect of genetic variants on protein function.

Abbreviation	Software name	Description	Source
SIFT	Sorting Inolerant From Tolerant	Predicts whether an amino acid substitution affects protein function based on the degree of conservation of amino acids in a sequence alignment.	Kumar et al.,[60] 2009
PolyPhen2	Polymorphism Phenotyping version 2	Predicts the possible impact of an amino acid substitution on the structure and function of a human protein.	Adzhubei et al.,[61] 2013
PhyloP	Phylogenetic *P*-values	Gives an estimation of the evolutionary conservation at individual alignment sites	Siepel et al.,[62] 2006
Grantham	Grantham	Predicts the distance between two amino acids at an evolutionary level	Grantham[63] 1974
GERP++	Genomic Evolutionary Rate Profiling version 2	Consists of two programs, *gerpcol* and *gerpelem*. The first estimates constraints introduced by substitutions for each column of the alignment; the second then identifies constrained elements that occur.	Davydov et al.,[64] 2010
CADD	Combined Annotation Dependent Depletion	A tool that combines multiple annotations into one value by contrasting variants using prediction and conservation tools.	Kircher et al.,[65] 2014

A complete list of prediction software is summarized by Richards et al., [66] 2015.

The American College of Medical Genetics and Genomics published guidelines for the interpretation of genetic variants in Mendelian disorders. However, these guidelines are generally not applicable for common complex disorders like AMD. Terms like pathogenic and likely pathogenic are usually not informative in complex disorders; more accurate proof comes from statistical (association) analyses that implicate a genetic variant in the disease. It is recommended to describe the variants that are linked to the disorder as 'risk alleles'.[66]

Association analyses of rare variants

When performing a large number of tests in association studies, the expected number of findings that cross the significance threshold of 0.05 will be considerable. Therefore, analyses with large number of variables should implement a correction for multiple testing.

Implementing such corrections, like Bonferonni or False Discovery Rate, can help reduce the number of false positive findings, but could consequently increase the number of false negative findings.[68] Effective strategies to analyze rare variants is to group them based on gene association, location in the genome, or functional characteristics in a gene-based burden or variance-component test.[4] In this way, correction can be applied to the number of genes interrogated instead of the number of variants.

In this thesis, WES was performed in a case-control cohort consisting of 2486 individuals. First we performed a single variant analyses, and could confirm the previously described common variants in *CFH* and *ARMS2* (**Chapter 9**). Single variant analysis had insufficient power to detect association with individual rare variants. Therefore, we next used gene-based burden tests, which accumulate the association of multiple rare variants per gene, to improve detection power. We used the combined multivariate and collapsing (CMC) method.[69] This method 'collapses' variants in a gene by recoding the variant status as present or absent. This binary recoding is done for rare and common variants separately. The model then evaluates the joint effect of the variants. This allowed us to observe a disease burden for the *COL8A1* gene. The disease burden was explained by 14 rare protein-altering variants in *COL8A1* which were found more often in cases (1.0%) than control individuals (0.4%). Unlike predefined gene-sets or variant-sets,[10,13] we were able to detect exome-wide rare variants. Unfortunately, our study did not have sufficient power to confirm the disease burden of previously reported genes (*CFH, CFI, TIMP3* and *SLC16A8*).[13]

Adding genetic data of additional family members or other cohorts to the dataset may increase the power to detect association, but could potentially be outweighed by problems of heterogeneity in disease state, environmental differences, or even aberrations in allele frequency between populations.[3] In **chapter 9** we combined our WES case-control dataset with that of the Rotterdam Study.[72,73] Both datasets were generated using the same exome enrichment kit, and to avoid discrepancies in calling between the two datasets we performed joint genotyping and recalibration of the files before quality control and annotation. In **chapter 3** we added family members to a case-control cohort in a binominal distribution model, while correcting for the shared genetic variation between the siblings. This allowed us to combine the two datasets and increase our sample size without creating an ascertainment bias.

Table 4: Different strategies of providing evidence to implicate genetic variants to disease phenotype.

Level	Class	Type
Gene level	**Genetic**	**Gene:** within a gene an excess of genetic variants are found predominantly in cases compared to controls.
	Experimental	**Protein interactions:** the protein interacts with other proteins previously implicated in the disease of interest (genetically or biochemically). **Biochemical function:** the protein performs a function shared with other known genes in the disease of interest, or consistent with the phenotype. **Expression:** the gene is expressed in tissues relevant to the disease of interest and/or is altered in expression in patients who have the disease. **Gene disruption:** the gene/ protein function is altered in individuals carrying candidate mutations. **Model systems:** animal or cell-culture models with a similarly disrupted copy of the affected gene show a phenotype similar to the human disease. **Rescue:** the cellular phenotype in patient-derived cells can be rescued by addition of the wild-type gene product.
Variant level	**Genetic**	**Association:** the variant is significantly enriched in cases compared to controls. **Segregation:** the variant is co-inherited with disease status within affected families. **Population frequency:** the variant is found at a higher frequency in study cohort compared to large population cohorts with similar ancestry to patients (consistent with the proposed inheritance model and disease prevalence).
	Bioinformatic	**Conservation:** the site of the variant displays evolutionary conservation consistent with deleterious effects of sequence changes at that location (like SIFT[60]). **Predicted effect:** variant is found at a location within the protein predicted to cause functional disruption (like PolyPhen2[61]).
	Experimental	**Gene disruption:** the variant significantly alters levels, splicing or normal biochemical function of the protein of the affected gene (in patient cells or *in vitro* model). **Phenotype recapitulation:** introduction of the variant into a cell line or animal model results in a phenotype that is consistent with the disease. **Rescue:** the cellular phenotype in patient-derived cells or model organisms can be rescued by addition of wild-type gene product or specific knockdown of the variant allele.

Table adapted from [66,70,71].

For complex disorders, large case-control studies are required to find a genetic association, in particular for rare variants. In smaller studies functional characterization of non-synonymous variants may help to assess causality of variants, and provide evidence that the gene is essential in a specific biological mechanism (**Table 4**). The bottle neck, however, of performing functional tests (*in vitro* or *in vivo)* is, that the experiments can require extensive hands-on work and time.[68] **Table 5** summarizes the rare genetic variants identified in the *CFH* gene, for which functional work was performed and the association was evaluated in the largest available AMD cohort.[13,17]

Only a few genetic variants are linked to the pathogenesis of AMD with strong genetic and functional evidence. There are also variants that are significantly enriched in AMD cases or controls individuals, but for which no functional evidence has been provided, and vice versa. This makes it difficult to interpret their effect on the disease pathogenesis. When generation of additional genetic evidence is not possible, for example due to a limited sample size or

when a variant is very rare, functional experiments can provide additional evidence on whether variants have an effect on the protein.

New sequencing methods are under development and are referred to as third-generation sequencing. These methods allow the detection of a single molecules, and optimized long-range sequencing.[74] These types of approaches will especially be useful for sequencing highly similar regions like *CFH* and *CFH-related* genes, or regions that have low coverage in current WES datasets, like *CFB/C2*.

Table 5: Genetic variants in *CFH* for which functional analyses were performed

Evidence	Variant in CFH	Genetic association	Functional implications (reviewed in [17])
Genetic and function	**p.Arg53Cys**	OR 22.54; p=0.001	No effect on FH serum levels, but reduced binding affinity, DAA and CA.
	p.Arg53His	OR 13.39; p=0.01	Low C3 serum level, reduced binding affinity, DAA and CA.
	p.Arg1210Cys	OR 20.28; p=8.91E-24	No effect on CA, but reduced binding affinity and in a partial FH deficiency
Genetic	**p.Gln950His**	OR 0.72; p=0.003	No effect on FH serum levels, does not affect CA and normal hemolytic activity. Slightly increased lysis in hemolytic assay.
	p.Asn1050Tyr	OR 0.36; p=5.92E-44	Abnormal C3 but normal FH levels in serum
Function	**p.Arg127His**	not significant	Reduced FH serum levels and impaired protein secretion.
	p.Val609Ile	not significant	Affects FH expression and resulted in decreased alternative pathway activity
No effect	**p.Gln400Lys**	not significant	No or only mild effect on plasma concentrations of FH, C3 and FB
	p.Ser890Ile	not significant	No effect on FH serum levels, does not affect binding affinity or CA and normal hemolytic activity.
	p. Thr956Met	not significant	No effect on FH or C3 plasma levels, no effect on expression or lysis.
	p.Val1007Leu	not significant	No effect on FH plasma levels, normal binding affinity and CA .
	p.Gly1194Asp	not significant	Normal serum levels for FH, FI and C3, slightly increased hemolytic lysis.

Genetic variants reported in multiple AMD case-control studies and available odds-ratio as reported Fritsche et al.,[13] 2016.. Abbreviations: CA = co-factor activity; DAA = decay accelerating activity

THE COMPLEMENT SYSTEM AND AMD

There are several lines of evidence to support a role of the complement system in the pathogenesis of AMD, both genetically[13] and functionally on the level of the retina, RPE and choroid.[75,76] In this thesis we used different functional measures of the complement system

to assess the impact of genetic variants on function. Furthermore, we performed genotype-phenotype correlation analyses between the location of an amino acid change in the protein for different complement-related disorders. In addition, we described a distinct macular phenotype in individuals carrying rare genetic variants in a complement gene.

Measuring the activity of the complement system in AMD

The complement system is an elaborate and intricately regulated network of interacting proteins that activate or inhibit each other, stabilize or cleave components to enhance or attenuate activity. Complement activation can be defined in several ways. One could measure the overall activity of the complement system by determining the total membrane attack complex (MAC) formation. However, formation of the terminal complex is not the only relevant functional outcome of complement system activity. Upstream is the C3 convertase that determines to what extent the initial trigger is amplified. Convertase-mediated cleavage of C3 releases C3a and induces a conformational change in the remaining fragment, named C3b. C3b fulfils different roles, including complement activation through tick-over, to driving the amplification loop, mediating phagocytosis and initiating the generation of the terminal pathway. C3b can be processed by components of the regulators of complement activation family (RCA) into iC3b (C3dg and C3f), and binding of the RCA to C3b also provides a binding site for FI to inactivate C3.[77] Over-activation or dysregulation of the complement system can be determined by measuring one or multiple of these components within the cascade.

Inactivation of C3 eventually leads to the components C3dg and C3f. C3dg can be further digested releasing C3g and C3d. The C3d/C3 ratio has proven to be a sensitive marker of complement activation, correcting for the individual variation in C3 levels.[78] In **chapter 2** we used the C3d/C3 ratio as a measure of complement activation.

In the presence of FH and FI, the C3 alpha chain is cleaved into three fragments (alpha 68- 46- and 43 kDa). These fragments can be visualized on a protein gel by using fluorescently labeled C3b on serum samples. The intensity of the 43kDa fragment (in relation to the alpha chain) can be used to assess cofactor activity efficacy. In **chapter 3**, we assessed complement activity by measuring C3b degradation in the presence complement inhibitors with or without rare variants.

Systemic complement activation can be measured in serum or plasma, derived from whole blood samples.[79-81] In **chapter 3** and **9** enzyme-linked immunosorbent assays to detect the levels of soluble proteins FH, FI, C3 and C9 or the terminal complement complex were used. Another outcome measure to assess the effectiveness of the complement system is to determine if it can lyse foreign cells (**chapter 9**). Erythrocytes are non-nucleated cell that lyse relatively easy when exposed to a sensitized antibody. The released hemoglobin level in the supernatants

are used as an outcome measure of lysis. On the other hand, lysis of nucleated cells is more challenging as these cells actively shed MAC. Using a cell line, like ARPE-19 cells we used in **chapter** 8, cell lysis can be measured via lactate dehydrogenase release and apoptosis.

It is hypothesized that local complement activation, rather than systemic activation, is of importance in AMD development. Local complement activation in the eye is usually determined by histological studies of ocular tissues from AMD patients.[76,82,83] To date, only one study measured local complement activation using aqueous humor of AMD patients.[84] The study showed a local increase of activation products Ba and C3a in neovascular AMD patients compared to control individuals. The up-regulation of activation products Ba and C3a was not significantly associated with AMD in systemic plasma levels, although a correlation was found between local and systemic activation products.[84] It is likely that the complement regulatory system of the eye is regulated separately from circulation. For example, membrane attack complex (MAC) deposits accumulate in the aging choriocapillaris, but its accumulation is not observed in other organs. The increased accumulation may cause thinning and degeneration of the choriocapillaris, which would then fail to remove cellular debris and allow drusen to form.[85] Further research is needed to elucidate the relation between local and systemic complement activation, although this is hampered by the difficultly of obtaining ocular tissue and fluid.

The effect of genetic variants in complement genes

It is important to understand the functional effect of genetic variants on the protein to assess relevance and causality. Knowing the relevance of a variant can optimize prediction of disease onset, help design preventative measures, allow early detection, and specify treatment options.

Complement factor H and complement factor I

In **chapter 3** we performed functional analyses to assess the effect of rare genetic variants in the *CFH* and *CFI* genes on the function of complement regulators FH and FI, respectively. For FH no aberrations in serum levels were observed compared in carriers compared to noncarrier individuals. In previous studies serum concentrations in carriers of rare *CFH* variants were found to reduced or normal compared to a reference.[86,87] This indicates that not all rare variants in *CFH* lead to lower FH levels, and that there are two types of mutations. Type 1 mutations cause low protein levels as a result of misfolding or degradation, whereas type 2 mutations result in reduced functionality with normal protein levels. For FI we observed a median reduction in serum levels for carriers of rare variants in *CFI (*p.Gly119Arg and *CFI* p.Leu131Arg). A recent study demonstrated that a large number of rare *CFI* variants are type 1 mutations, resulting in lower FI levels.[67]

We also measured the C3b degradation ability for individuals carrying *CFH* or *CFI* variants, and observed that carriers of rare variants in *CFH* and *CFI* had a lower capacity to degrade C3b

compared to noncarriers. FH serum levels remained stable, suggesting that *CFH* variants affect complement activation independent of FH serum levels by its inability to properly serve as a cofactor in the cleavage of C3b to inactive C3b. This finding might be explained by the variants' location at the N-terminus, where a C3b-binding site is located. For FI it was previously shown that both expression and secretion of mutant FI protein were reduced compared with wild-type protein, and that it were the lower FI levels that led to reduced C3b degradation.[11]

Complement component 9

For many variants in complement genes, functional data is already available, scattered throughout literature and organized in **chapter 1B**. Unfortunately, there is little known for complement component C9. Therefore, we evaluated the functional effect of rare variants identified in *C9* in **chapter 4**. We identified elevated C9 serum levels for carriers of rare variants in the *C9* gene (p.Met45Leu, p.Phe62Ser, p.Pro167Ser and p.Ala529Thr). However, no increase in serum concentration for the terminating complement complex between carriers and noncarriers was observed. The MAC is dependent on the incorporation of C9. We hypothesized that elevated levels of C9 lead to enhanced MAC deposition and damage to the retinal cells. However, we could not detect any increase in C9-dependent lytic activity of patient sera. More surprisingly, the p.Phe62Ser and p.Pro167Ser mutant proteins caused decreased lysis, which was contrary to our expectations. We did note a tendency of the p.Pro167Ser mutant protein to spontaneously aggregate and an increased polymerization of p.Phe62Ser and p.Pro167Ser mutant proteins. Future experiments need to be performed to evaluate the role of *C9* p.Pro167Ser in AMD pathogenesis, for example, by determining the effect of protein aggregates on cells by measuring stress-related markers or by visualizing C9 expression on Western blot to see if polymerization of the protein occurs intracellular or extracellular.

Phenotypes of AMD patients carrying complement variants

Genetic variants in the *CFH* gene result in a specific AMD phenotype.[19,88-91] Initially, it was observed that the common risk variant p.Tyr402His associated with the AMD subtype cuticular drusen.[89,90] Later, also rare genetic variants in *CFH* were described to show distinct phenotypic features, including increased drusen and appearance of cuticular drusen.[19,91]

Ferrara and colleagues characterized 143 individuals based on fundus images, of which half (n=62) carried the rare variant *CFH* p.Arg1210Cys. For these individuals it was reported that carriers of the *CFH* p.Arg1210Cys variant had high macular drusen scores and were more likely to have advanced AMD compared than those without the variant.[88] It should be noted that the noncarrier group contained more individuals without AMD, namely 28.4% (23/81), than the carrier group with 4.8% (3/62).

In **chapter 6** we compared phenotypical characteristics of 51 carriers of rare *CFH* variants with 102 age-matched noncarriers. Both groups consisted solely of individuals affected by AMD. For *CFH* carriers we observed an increased number of drusen around the macula and nasal to the optic disc. In addition, an association with the presence of crystalline or calcified drusen was found for *CFH* carriers. Presence of this type of drusen increases the risk of developing geographic atrophy.[92,93] *CFH* carriers may develop geographic atrophy more often compared to choroidal neovascularization, as we observed for rare variant carriers of *CFI, C3*, and *C9* variants in **chapter 2**. Unfortunately, the sample size of this study was too small to draw definitive conclusions on the association of rare variants and particular AMD subtypes.

Based on the findings we and others[88] reported it is unlikely that carriers can be discriminated from noncarriers based solely on phenotypical characteristics. Other patient characteristics, like an early age at onset of symptoms, presence of cuticular drusen, and a positive family history for AMD can aid ophthalmologists to select patients for genetic screening.[18,19,33,36,88,94]

Complement variants in AMD and renal disorders

Dysregulation of the complement system has been associated with very different clinical outcomes. This group of disorders, known as 'complementopathies', is characterized by over-activation of the complement system. Inhibition of the complement system in these diseases may halt or reverse the disease process. Examples of complementopathies include paroxysmal nocturnal hemoglobinuria (PNH), cold agglutinin disease (CAD), atypical hemolytic uremic syndrome (aHUS), and C3 glomerulopathy (C3G).[95] It was recently suggested that AMD should also be classified as a complementopathy.[96]

We focused on protein-altering variants in complement genes that are reported to cause AMD but also renal diseases aHUS and C3G.[97,98] aHUS and C3G are rare disorders resulting in acute and lethal renal failure. Furthermore, aHUS manifests at an earlier age (90% of the patients is younger than 55)[99] than advanced AMD, which is most often observed in individuals above the age of 65.[100] It is interesting how such different disorders in age of onset, tissue type and lethality can both result from genetic alterations in the complement system.

The location of a variant can influence the properties of a given protein, which can be visualized and predicted from a three dimensional structure. Amino acid changes can have an effect on activity, aggregation, stability, binding, assembly or arrangement of the protein domains.[101] In AMD, an enrichment of protein-altering variants has been reported in the N-terminal (SCR1-4) and C-terminal (SCR19-20) short consensus repeats (SCR) domains of FH, in the serine protease domain of FI, and in the MG1 and MG2 domains of C3.[17,102] Each of these domains is involved in co-factor activity mediated cleavage of C3b by FH and FI. Variants in these domains could therefore interfere with proper complement regulation. In **chapter 8** we observed an

increased number of protein-altering variants in the SCR3, SCR5 and SCR7 domains of FH, the scavenger receptor cysteine-rich (SRCR) domain of FI, and the MG3 domain of C3 in AMD patients compared to aHUS and C3G. For aHUS and C3G patients we observed an increased number of variants in the C-terminus of FH, and in the serine protease domain of FI.

FH domains SCR 3, 5 and 7 are part of both the full length FH and the alternatively spliced factor H-like protein 1 (FHL-1). FHL-1 has the same functions as FH as it can bind C3b, interact with FI, and bind to Bruch's membrane using the glycosaminoglycans binding site located in SCR7. However, FHL-1 can, due to its small size, regulate complement within Bruch's membrane and drusen.[103-106] We confirm that variants in the first domains of FH (up to SCR7) play a more important role in AMD pathogenesis, while the C-terminus is more important in renal disorders. The SRCR domain in FI does not contain any known interaction sites with cofactors, however, FI serum levels of AMD individuals carrying low frequency variants in the SRCR domain were reported as reduced. These reduced levels consequently impair normal FI function.[11,25,107] The serine protease domain (together with the FIMAC domain) contains the binding sites important for C3b and C4b degradation[108,109]. Variants found in the serine protease domain could interfere with proper cofactor activity as the domain cannot be stabilized for proteolytic activity. The MG3 domain of C3 is the only domain in the β-ring that undergoes rearrangement during conversion from C3 to C3c. Variants in this domain could possibly interfere with this rearrangement.

Genetic alterations in the complement system have been linked to aHUS, C3G, and AMD. However, individuals with rare or low frequency variants in the complement system present phenotypic characteristics of only one disorder.[15,16] There are a number of hypotheses that could explain this observation. First, it is possible that the genetic burden in the complement system is higher in aHUS compared to AMD.[110] In **chapter 8** we observed three times as many rare genetic variants in aHUS/C3G compared to AMD. Second, AMD associated variants are generally common, and many variants reside is genes other than *CFH*, *CFI* and *C3*.[13] Therefore, AMD is a result of a combination of common and rare variants in many genes, and is not only attributed to rare variants in the complement systm. Third, a different distribution of variants was observed in protein domains involved in AMD compared to renal disorders, suggesting that a genotype-phenotype correlation exists, at least for some of the variants. Fourth, genetic predisposition is not always enough to develop aHUS or AMD, but can act to exacerbate other triggering events. For aHUS these triggers include pregnancy, infections, and immunosuppressive drugs.[111] For AMD, oxidative stress in the retina may act as a local trigger, among others.

Because of the clear involvement of the complement system in both diseases, complement-targeting therapies are actively being developed. For aHUS, successful implementation of a complement-inhibiting drug, Eculizumab, has already been achieved. Before approval

of Eculizumab in 2013 for treatment of aHUS,[112] patients had to undergo kidney and liver transplantation to normalize complement regulation. No complement inhibiting therapy is currently approved for AMD, but several clinical trials are ongoing to determine the effectiveness of these therapies.

COMPLEMENT INHIBITORS

Numerous trials using complement inhibiting agents, including protease inhibitors, antibodies against complement components, complement regulators or inhibitors, and anaphylatoxin receptor antagonists are ongoing or have been performed in AMD. From a patients perspective, the most important outcome of a therapy is the maintenance of visual acuity at follow-up. In a clinical trial setting other parameters can be used as trial outcomes, including visual function, quality of life, prevention of AMD progression (non-advanced to advanced AMD), and lesion size of the geographic atrophy.[113]

Clinical trials with complement inhibitors so far have produced disappointing results in AMD (**Table 6**). Some trials ended early for not meeting the interim criteria, like AL-78898A, and others reported only minimal improvement in visual acuity, like Eculizumab. These initial results suggest that we have insufficient knowledge on what role the complement system plays in AMD development. Until we know more about the exact mechanism, we are unable to precisely determine which subgroup of patients would benefit, which component in the cascade we should target, at which stage of the disease patients should be treated, and whether local or systemic administration would be most effective.

Each AMD patient is different in genetic background, age at onset of disease symptoms, current AMD stage and burden of environmental factors. More importantly, deregulation of the complement system may not be the underlying cause for developing AMD in all patients. It should be noted that there is a great range in complement levels, and that these are influenced by many factors including age of the individual, BMI, smoking status, AMD stage and genetic variation.[80,81,114-119]

Most complement inhibitors developed for treatment of AMD-associated geographic atrophy focus on the alternative pathway of the complement system, like the C3 and C5 convertases (**Table 6**). An alternative to this strategy would be to focus on the complement regulators such as FI and FH instead. Synthesized or plasma derived FI or FH could be used to treat AMD patients that show deficiencies for complement regulators.[120] Rare variants in the *CFH* and *CFI* gene can lead to reduced serum levels, and supplementation may be beneficial for these types of complement dysregulation.[10,11,15,25,36,67,86,121] To support this view, a synthesized short form of

FH showed promising results in model organisms, and is also considered as treatment option in FH associated complement dysfunction such as AMD.[122-125]

The MAHALO study[126] is a phase II clinical trial with Lampalizumab, an antibody directed against complement factor D. In this study, a reduction of lesion growth and loss of visual acuity was observed. Sub-analysis suggested that the reduction in lesion growth was mainly driven by the strong reduction seen for a common *CFI* risk allele group. Moreover, the *CFI* risk allele was linked to reduced FI expression in liver tissue. Unfortunately, more recently Phase III results, showed no reduction of mean change in lesion size at the primary endpoint (**table 6**). It therefore remains unclear whether carriers of this common CFI risk variant would benefit more from complement-inhibiting treatments.

We estimate that 10-20% of all AMD patients carry rare variants in the *CFH* and *CFI* genes. In **chapter 3** we report that carriers of rare *CFH* and *CFI* variants have an impaired ability to regulate complement activation. We hypothesize that these patients may benefit more from complement-inhibiting therapy than patients with AMD in general. Further research focused on the subgroup of AMD patients which are burden by rare genetic variants in *CFH* and *CFI* could help improve personalized treatment.

Clinical trials for dry AMD currently focus on patients with advanced geographic atrophy. The geographic atrophy results from a slow and gradual degeneration of retinal pigment epithelium and photoreceptor cells in the central retina. Once these lesions appear they lead to the complete loss of cell structure.[127] Complement inhibiting trials currently aim to slow the lesion growth, and cannot restore the cell structure that has already been lost. However, atrophy formation is a complex process in which inflammation and cell stress play a role over a long period of time. Once the complement system is triggered, it acts through different mechanisms. Loss of retinal pigment epithelium and Bruch's membrane function increases access of complement proteins to the outer neural retina. Impaired or reduced expression complement regulators leads to complement attack on retinal pigment epithelium cells and photoreceptors. An increase in anaphylatoxins, opsonization and MAC formation, together with increased presence of phagocytes, promotes further loss of photoreceptors and retinal pigment epithelium.[128] Stopping any of these individual processes may therefore not be sufficient to halt GA progression. Possibly complement inhibition therapy would be more effective before the onset of atrophy, preventing the formation of atrophy in the first place.

Most trials inhibiting complement target local complement activation rather than systemic activation (Table 6).[129] Local administration has several advantages, as it will not only prevent disruption of the systemic immune system, which increases susceptibility of infections, but it also has the advantage of direct administration to the affected tissue. Local administration

of the drug is done via intravitreal injection. Important in that regard is that it is essential that the drug has the ability to diffuse through the BM to be able to reach the retinal pigment epithelium, Bruch's membrane and choroid.[129] Ideally, invasive procedures like intravitreal injection sould be replaced by less invasive options like topical administration.

DIAGNOSTIC PERSPECTIVE

Given the impact on quality of life and the limited treatment options for AMD, there is considerable interest in predicting the effect of genetic variants on disease onset and progression. A predictive model would allow taking appropriate preventative steps and further aid personalized and precision medicine.

A reliable risk model for AMD incorporates genetic markers in addition to environmental triggers, baseline phenotype and demographic information.[131] The best performing models to predict AMD progression include AMD risk genes, smoking status, body mass index, and baseline AMD phenotype.[132-134] These data are relatively easy to obtain, in contrast to dietary factors, systemic biomarkers and complement measurements, which often need extensive laboratory workup or complex questionnaires. The models should include information on common genetic variants associated with AMD.

Such genetic information can be obtained using a targeted sequencing assay. With such an assay, single common and rare variants, next to the complete sequencing of a set of genes, such as genes causing AMD-mimicking macular dystrophies and AMD-associated complement genes, can be analyzed simultaneously. This genetic test would enable the detection of patients with AMD-mimicking macular dystrophies, which can be clinically challenging to differentiate from AMD (**chapter 7**). The contribution of rare complement variants in a prediction model is limited due to their low population frequency, but they are informative for variant- or gene-specific treatment.[132] Seddon and colleagues[135] recently published such a risk calculator that allows clinicians to enter patient specific genetic and environmental information to calculate a risk score. This risk calculator includes eight common genetic variants and two rare genetic variants (*CFH* p.Arg1210Cys and *C3* p.Lys155Gln), along with information on age, sex, education, BMI, smoking status and macular phenotype.

Evaluating the effect of novel rare variants will be challenging, even if these variants reside in genes associated with AMD. Looking up the minor allele frequency in large population databases (like ExAC or gnomAD),[54] and consulting functional information from public databases can help assess the impact of an identified variant. Ultimately, incorporation of biological information into a clinically useful risk model is required to properly assess the impact of a specific rare

Table 6: Complement inhibitors in clinical trials for advanced AMD with geographic atrophy.

Agent	Mechanism of action	Administration	Stage (Clinicaltrials.gov Identifier)	(Preliminary) results
Lampalizumab	interacts with complement factor D	Intravitreal injection	Phase III – ongoing (NCT02247531, NCT02247479 , and NCT02745119)	Phase II reported a reduction in lesion growth.[126] Phase III (SPECTRI, CHROMA and OMASPECT) is ongoing. SPECTRI did not meet its primary endpoint of reducing mean change in lesion size (press release by Genentech; http://www.roche.com).
Eculizumab	inhibits complement factor C5	Intravenous	Phase II – completed (NCT00935883)	Did not decrease the growth rate of GA significantly.[130]
LFG316	interacts with complement factor C5	Intravitreal injection	Phase II – completed (NCT015275000) Phase II – ongoing (NCT02515942)	No results reported
ARC1905 (Zimura)	interacts with complement factor C5	Intravitreal injection	Phase I – completed (NCT02686658)	No results reported
AL-78898A (POT-4)	inhibits cleavage complement factor C3	Intravitreal injection	Phase II – terminated (NCT01603043)	Efficacy analysis was not conducted due to the termination of the study prior to efficacy endpoints and insufficient patients. (not published)
APL-2	inhibits cleavage complement factor C3	Intravitreal injection	Phase II – ongoing (NCT02503332)	Reduction in the rate of geographic lesion growth over 12 months according to Apellis Pharmaceuticals (http://www.apellis.com)
CLG561 (with and without LFG316)	Inhibits properdin	Intravitreal injection	Phase II – ongoing (NCT02515942)	No results reported

Includes only clinical trials that successfully completed phase I.

variant. In **chapter 3** and **4** we performed functional analyses for a selected number variants found in *CFH, CFI, C3*, and *C9*. Systematical functional analyses of all variants identified in these genes will help interpret the consequences of rare coding variants on protein function. Functional analyses include measurements of circulating serum or plasma levels, or evaluating the effect of a variant in cell-based or animal models. For example, comparing systemic serum levels of variant carriers to a reference,[67] cloning variants in vectors and expressing these in cell lines to assess altered activity, or injecting human mRNA in a zebrafish model to assess changes in vascular architecture in the eye as preformed previously.[136] Other functional data that can be systematically integrated in a future prediction model include: gene expression, DNA methylation, proteomics, metabolomics, transcriptomics, or a complement-directed assay.

The commercial company 23andMe offers genetic testing including health reports on a variety of treatable and untreatable diseases. It was very recently announced that AMD will be added to the list of conditions for which can be screened.[137] The test will screen for common variants *CFH* p.Tyr402His and *ARMS2* Ala69Ser. Although these variants confer the highest common risk for AMD,[13] they are insufficient to make a reliable prediction, especially when considering that 50 other genetic variants have been also associated with AMD,[13] including many with a protective effect. The first prediction model by Gold and collaegues[138] tested variants in two genes, namely *C2/CFB* and *CFH*, and reported a correct prediction in 74% of the affected individuals and only 56% of the controls. More recently[132] it was shown that prediction tests became more reliable with a larger number of common genetic variants included. Furthermore, variables like age, smoking status, and BMI are essential for an accurate prediction. 23andMe's health report will inform customers on their risk to develop disorder, an explanation of what the results mean, an overview of the condition, other factors that may influence risk, and suggests next steps. Keeping in mind that, at the moment, there is no preventative treatment for AMD other than to stop smoking and taking dietary supplements, there seems to be no harm (but also no significant advantage) in direct-to-consumer screening currently as offered by 23andMe.

In this thesis, we described the identification of number of rare genetic variants residing in the complement system, extracellular matrix and inherited macular dystrophy genes using case-control and family-based analyses. These methods were effective to identify novel rare genetic variants, but ultimately supportive information from biological experiments is required to properly assess the impact of specific rare variants. Systematical functional analyses of rare genetic variants within a gene of interest will help interpret the consequences of rare coding variants on protein function in general. Functional analyses in combination with genetic information should be integrated in a diagnostic setting to reliably asses rare variant implications. Rare genetic variants have functional consequences that help us understand the underlying disease mechanisms. A better understanding of the disease mechanism as a whole

is needed to optimize treatment options. Knowing which role rare genetic variants play may therefore help us answer the questions whom to treat, what the drug should target, and how the drug should be administered.

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11

SUMMARY
SAMENVATTING
LIST OF PUBLICATIONS
CURRICULUM VITAE
DANKWOORD

SUMMARY

Age-related macular degeneration (AMD) is a disease affecting the central retina as result of a complex interaction of environmental and genetic risk factors. AMD is the most common cause of irreversible vision loss in the Western world. It was previously hypothesized that a common disease, like AMD, would be caused by common genetic variants. However, after uncovering 19 loci associated with the disease, a large fraction of the heritability remained unexplained. Rare genetic variants, defined as genetic changes with a minor allele frequency below 1% in the population, can have large effects on disease and were proposed to account for the 'missing' heritability. The aim of this thesis is to further elucidate the role of rare genetic variants in AMD pathogenesis. We hypothesize that a proportion of the missing heritability in AMD can be explained by rare highly penetrant variants. We aim to identify novel genetic causes of AMD, and to understand their role in the disease mechanism of AMD.

In **chapter 2** we reported the identification of rare genetic variants *CFI* p.Gly119Arg, *C9* p.Pro167Ser, and *C3* p.Lys155Gln, which were previously associated with AMD, in 22 densely affected AMD families. Despite the strong association with AMD in case control association analyses, these rare variants did not segregate with the disease phenotype in our families. The presence of one of these rare variants was associated with a higher familial occurrence, as more than half of all patients who carried such a rare variant reported a positive family history for AMD. Clinically, the initial onset of symptoms in carriers of these rare variants occurred an average of six years earlier than in non-carriers. In addition, these genetic rare variants were more prevalent among patients with advanced AMD and geographic atrophy than in patients with neovascular AMD. Our finding emphasized the importance of counseling patients and family members to increase awareness and enable early detection of the disease.

In **chapter 3** we continued our analysis into the 22 families we described in chapter 2 in search for additional rare variants in complement genes *CFH, CFI, C3*, and *C9*. We identified 6 additional rare genetic variants. Although the identified variants were associated with AMD disease status and highly prevalent within these families, perfect segregation with the disease phenotype was not observed. By analyzing the effect of rare variants on protein expression, we found that carriers of *CFH* and *C3* variants have normal serum FH concentration, *CFI* carriers (p.Gly119Arg and p.Ser193Leu) have reduced FI serum concentration, and *C9* p.Pro167Ser carriers have increased C9 serum concentration. Furthermore, serum samples from carriers of rare variants in *CFH* and *CFI* revealed a diminished ability to degrade C3b, suggesting that the variants result in impaired complement regulation. We hypothesize that carriers of rare variants in *CFH* and *CFI* are less able to inhibit complement activation and may therefore benefit more from complement-inhibiting therapy than AMD patients in general.

In **chapter 4** we evaluated the functional effect of seven rare variants in *C9* identified in our AMD cohort. We identified elevated C9 serum levels for carriers of rare variants in *C9* (p.Met45Leu, p.Phe62Ser, p.Pro167Ser and p.Ala529Thr), however no increase in serum concentration for the terminating complement complex between carriers and noncarriers was observed. Formation of the membrane attack complex (MAC) depends on the incorporation of C9. We assumed elevated concentrations lead to enhanced MAC deposition and damage to retinal cells. However, we could not detect any increase in C9-dependent lytic activity of patient sera. More surprisingly, the p.Phe62Ser and Pro167Ser variants caused decreased lysis which was contradictory to our expectations. We did note increased polymerization for p.Phe62Ser and a tendency of the p.Pro167Ser mutant to spontaneously aggregate. Future research is needed to evaluate the role of *C9* p.Pro167Ser in AMD pathogenesis.

In **chapter 5** we explored the geographical occurance of seven rare genetic variants that were reported to be independently associated with AMD by the International AMD Genomics Consortium. We observed that two rare AMD-associated variants in the *CFH* gene (rs121913059 [p.Arg1210Cys] and rs35292876) deviated in allele frequency among different geographical regions. As expected, risk estimates of each of the seven rare variants were comparable across geographical regions. Our findings emphasize that personalized treatment aimed at rare variants with functional effects may only be applicable to specific populations where these variants are sufficiently common.

In **chapter 6** we compared the phenotypical characteristics of patients with AMD carrying a rare variant in the *CFH* gene to those without such a rare variant. We observed an increased number of drusen deposition in rare variant carriers, around the macula and nasal to the optic disc. Furthermore, rare variant carriers were graded more often as late atrophic AMD (57.1%) than noncarriers (28.1%). These phenotypical characteristics could guide ophthalmologists in selecting genetic tests. When limiting the analyzed variants to those that were confirmed to be pathogenic by case-control or functional studies, the associations between rare variant carriers and phenotypical characteristics became more pronounced. This emphasizes the importance of confirming the clinical significance of rare genetic variants.

In **chapter 7** we aimed to evaluate the occurrence of AMD-mimicking dystrophies in our cohort of 183 cases diagnosed with intermediate or late geographic atrophy AMD. Using whole exome sequencing we evaluated rare genetic variants found in 19 genes associated with autosomal dominant and recessive macular degenerations mimicking AMD. A mutation in *PRPH2* was identified (c.424C>T, p.Arg142Trp) in three individuals. This mutation is reported to be causal for central areolar choroidal dystrophy (CACD). Phenotypically there are strong similarities between CACD and AMD and based on images alone these patients can be easily overlooked and misdiagnosed. It is increasingly important to correctly diagnose patients with macular

degeneration with respect to inclusion in clinical trials and for future treatment. Based on our results genetic screening of the *PRPH2* gene is recommended to exclude AMD-mimicking dystrophies.

In **chapter 8** we compared the low frequency variants found in complement genes *CFH, CFI,* and *C3* for 866 renal diseases patients, diagnosed with atypical hemolytic uremic syndrome (aHUS) or C3 glomerulopathy (C3G), and 697 AMD patients. Analyzing the domains in which low frequency variants cluster, combined with structural information, can help assess which domains are of functional importance. Such insight may help understand the way in which complement activation is responsible for two seemingly different disease phenotypes. Genotype-phenotype correlations between the disease groups identified a higher frequency of protein-altering variants in SCR20 of FH and in the serine protease domain of FI for aHUS/C3G patients. For AMD, the N-terminus of FH, especially the SCR3, SCR5 and SCR7 domains, contained more protein altering variants, in addition to the SRCR domain in FI, and MG3 domain of C3. For FH, our findings corroborate existing data that shows that FH interacts with the glomerular endothelium via the C-terminus while the N-terminus of FH is involved in C3b regulation in AMD. Alterations in FI's SRCR domain are likely to affect expression and thus overall activity, while FI changes related to renal disease are involved in structural elements around the active site. For C3, alterations located the MG domains that interact with FH may hamper breakdown of C3. We observed a significant overlap in variants between aHUS/C3G and AMD, however there is a distinct clustering of variants within specific domains.

In **chapter 9** we analyzed whole-exome sequencing information of 1125 AMD cases and 1361 controls. Using single variant analysis we confirmed the association of common variants in *CFH* and *ARMS2*. Furthermore, we used gene-based burden tests called combined multivariate and collapsing (CMC), which accumulates the association from multiple rare variants per gene. Using CMC, we observed a disease burden for the *COL8A1* gene driven by 14 rare protein altering variants. Staining of mice retina demonstrated that COL8A1 localizes at Bruch's membrane and is slightly expressed in the outer plexiform layer of the retina. Our findings suggest that protein-altering variants in *COL8A1* could alter the integrity of Bruch's membrane, thereby contributing to the accumulation of drusen and the development of AMD.
In conclusion, evaluating the effect of novel rare variants is challenging, even if these variants reside in genes previously associated with AMD. In this thesis, we described the identification of number of rare genetic variants residing in the complement system extracellular matrix and AMD-mimicking macular dystrophy genes using case-control and familial-based analyses. These methods were effective to identify novel rare genetic variants but ultimately supportive information from biological experiments was required to properly assess the impact of a specific rare variant. Systematical functional analyses of rare genetic variants within a gene of interest will help interpreting the consequences of rare coding variants on protein function

in general. Rare genetic variants have functional implications that help us understand the underlying disease mechanisms. A better understanding of the disease mechanisms as a whole is needed to optimize treatment options. Knowing which role rare genetic variants play may therefore help us answer the questions: whom to treat, what the drug should target and how the drug should be administered.

SAMENVATTING

Leeftijdsgebonden maculadegeneratie (LMD) is een oogaandoening die de retina (beter bekend als het netvlies) aantast. Slijtage (degeneratie) van het netvlies kan leiden tot een verlies van gezichtsscherpte. LMD wordt veroorzaakt door een complexe interactie van omgevingsfactoren (zoals roken en voeding) en genetische risicofactoren. Deze ouderdomsziekte is de meest voorkomende oorzaak van verlies van het gezichtsvermogen in de westerse wereld.

Voorheen werd gedacht dat een veel voorkomende ziekte als LMD alleen veroorzaakt kon worden door veelvoorkomende genetische varianten. Echter, na het ontdekken van 19 genetische loci die betrokken zijn bij het ontstaan van de ziekte, kon toch een groot deel van de erfelijkheid niet worden verklaard. De hypothese werd daarom gesteld dat ook zeldzame genetische varianten de kans op het ontwikkelen van een veelvoorkomende ziekte kunnen vergroten. Een zeldzame variant wordt gedefinieerd als een genetische verandering met een allel frequentie lager dan 1%. Dit getal geeft aan hoe vaak een genetische verandering in een populatie voorkomt. Het doel van dit proefschrift is om verder te ontrafelen welke rol deze zeldzame genetische varianten spelen bij het ontstaan van LMD. We denken dat een deel van de ontbrekende erfelijkheidsfactoren in LMD mogelijk verklaard kan worden door zeldzame varianten. Met dit proefschrift willen we de genetische oorzaken van LMD verder identificeren en hun rol in het ziektemechanisme van LMD beter begrijpen.

In **hoofdstuk 2** rapporteerden we de identificatie van zeldzame genetische varianten *CFI* p.Gly119Arg, *C9* p.Pro167Ser en *C3* p.Lys155Gln in 22 families waar bij meerdere familieleden LMD was vastgesteld. Al eerder was aangetoond dat deze genetische varianten een rol spelen bij het ontstaan van LMD. Met behulp van een patiënt-controle associatie analyse bevestigden we dat deze varianten inderdaad risico geven op het ontstaan van de ziekte. Echter, de varianten erfden in de families niet volledig over met de ziekte, ondanks de sterke associatie met LMD. De aanwezigheid van een zeldzame variant leek wel invloed te hebben op het voorkomen van LMD binnen deze families. Meer dan de helft van alle patiënten met een dergelijke zeldzame variant had een positieve familiegeschiedenis voor LMD. Klinisch gezien werden symptomen bij dragers van deze varianten gemiddeld zes jaar eerder opgemerkt dan bij niet-dragers. Daarbij kwamen deze genetisch varianten vaker voor bij patiënten met gevorderde LMD in combinatie met geografische atrofie (droge LMD) dan bij patiënten met neovasculaire (natte) LMD. Onze bevindingen benadrukken het belang van het op tijd inlichten van familieleden van LMD-patiënten over hun mogelijke risico op LMD. Zodoende kan men bij de familieleden het bewustzijn vergroten en vroege opsporing van de ziekte mogelijk te maken.

In **hoofdstuk 3** hebben we onze analyse van de 22 families uit hoofdstuk 2 doorgezet. We zijn op zoek gegaan naar andere zeldzame varianten in genen uit het complementsysteem (namelijk

CFH, *CFI*, *C3* en *C9*). Het complementsysteem maakt onderdeel uit van het lichaamseigen afweer systeem. Binnen de families identificeerden we nog zes zeldzame genetische varianten in genen van het complementsysteem. Hoewel de geïdentificeerde varianten geassocieerd waren met LMD en veel voorkamen binnen deze families, werd geen perfecte overerving van de varianten met het ziektebeeld waargenomen. Door het effect van zeldzame varianten op eiwitniveau te analyseren, ontdekten we bij de dragers van *CFH* varianten normale serum FH-concentraties. Voor *CFI*-dragers (p.Gly119Arg en p.Ser193Leu) vonden we een verminderde FI serumconcentratie en voor *C9* p.Pro167Ser was de C9 serumconcentratie toegenomen. Bovendien toonden serummonsters van dragers van zeldzame varianten in *CFH* en *CFI* een verminderd vermogen om C3b af te breken. Dit suggereert dat de varianten resulteren in verminderde complementregulatie, dat wil zeggen dat de afweer tegen infecties en het opruimen van ziekmakende cellen verstoord is. We concluderen dat dragers van zeldzame varianten in *CFI* en *CFH* minder in staat zijn om dit mechanisme te remmen en daardoor waarschijnlijk meer baat zullen hebben bij complement remmende therapie dan LMD-patiënten in het algemeen.

In **hoofdstuk 4** onderzochten we het effect van zeven zeldzame genetische varianten in het *C9* gen op de functie van het C9 eiwit. In het complementsysteem vormt C9 met meerdere eiwitten een complex (genaamd het 'membrane attack complex' (MAC)) dat gaten kan boren in membranen van ziekmakende cellen om deze op die manier op te ruimen. In ons onderzoek toonden we verhoogde C9 serumwaarden aan bij dragers van zeldzame varianten in *C9* (p. Met45Leu, p. Phe62Ser, p.Pro167Ser en p.Ala529Thr). Er werd echter geen toename in serum concentratie waargenomen in de vorming van het MAC complex tussen dragers en niet-dragers. We veronderstelden dat verhoogde C9 concentraties zouden kunnen leiden tot verhoogde MAC vorming en uiteindelijk schade aan de netvliescellen. We konden echter geen toename van de C9 afhankelijke lytische activiteit (het boren van een gat) detecteren in cellen die werden blootgesteld aan serum van deze patiënten. De *C9* p.Phe62Ser en p.Pro167Ser varianten veroorzaakten een verminderde lytische activiteit, wat tegengesteld was aan onze verwachtingen. We vonden een verhoogde polymerisatie voor p.Phe62Ser en de neiging van de p.Pro167Ser-variant om spontaan te aggregeren (misvouwen en klonteren). In de toekomst is aanvullend onderzoek nodig om de rol van *C9* p.Pro167Ser in het ontstaan van LMD op te helderen.

In **hoofdstuk 5** hebben we onderzocht hoe vaak zeven zeldzame genetische varianten voorkomen in verschillende populaties. Voorheen was door het International LMD Genomics Consortium gerapporteerd dat deze zeven varianten geassocieerd zijn met LMD. We zagen dat de allel frequenties van twee LMD-varianten in het *CFH* gen (rs121913059 [p. Arg1210Cys] en rs35292876) afweken tussen verschillende geografische regio's. Zoals verwacht was het risico op LMD voor elk van deze zeven varianten vergelijkbaar binnen de geografische regio's.

Onze bevindingen benadrukken dat het voorkomen van zeldzame varianten kan verschillen per populatie, en dat in de toekomst in andere populaties mogelijk nieuwe zeldzame varianten kunnen worden ontdekt. Ook zal persoonsgerichte behandeling gericht tegen specifieke zeldzame varianten mogelijk alleen toegepast kunnen worden in populaties waar deze varianten vaak genoeg voorkomen.

In **hoofdstuk 6** hebben we de klinische kenmerken beschreven van patiënten met LMD met een zeldzame variant in het *CFH* gen. We hebben deze groep vergeleken met patiënten zonder een dergelijke zeldzame variant. Bij dragers van de zeldzame varianten zagen we een verhoogd aantal drusen (ophopingen van kalk, kristallen en afvalproducten) rond de macula en nasaal (neuszijde) van de oogzenuw. Bovendien hadden de dragers van zeldzame varianten vaker het eindstadium geografische atrofie (droge LMD; 57,1%) dan niet-dragers (28,1%). Wanneer er alleen gekeken werd naar dragers van varianten die de functie van het eiwit aantasten, dan waren deklinische kenmerken nog uitgesprokener. De klinische kenmerken van *CFH* dragers kunnen oogartsen helpen in het selecteren van patienten voor wie het aanvragen van een genetische test zinvol kan zijn.

In **hoofdstuk 7** wilden we het voorkomen van erfelijke macula dystrofieën bekijken die erg lijken op LMD. Dit hebben we gedaan in een groep met 183 LMD-patiënten met kenmerken van intermediair LMD of geografische atrofie (droge LMD). We maakten gebruik van whole exome sequencing, een techniek waarbij DNA snel uitgelezen kan worden. We evalueerden zeldzame genetische varianten uit 19 genen geassocieerd met autosomaal dominante en recessieve macula dystrofieën die op LMD kunnen lijken. Een mutatie in *PRPH2* (c.424C> T, p. Arg142Trp) werd geïdentificeerd bij drie personen. Deze mutatie is verantwoordelijk voor het ontstaan van centrale areolaire choriodale dystrofie (CACD). De klinische kenmerken van CACD en LMD komen sterk overeen. Op basis van netvliesfoto's kunnen deze patiënten gemakkelijk verkeerd worden gediagnosticeerd. Het wordt steeds belangrijker om patiënten met maculadegeneratie correct te diagnosticeren voor deelname aan klinische studies naar nieuwe behandelingen. Onze aanbeveling is om patiënten met droge LMD genetisch te testen voor het *PRPH2*-gen om op deze manier CACD uit te sluiten.

In **hoofdstuk 8** vergeleken we de varianten gevonden in complement genen *CFH, CFI* en *C3* bij 866 patiënten met nierziekten, gediagnosticeerd met atypisch hemolytisch uremisch syndroom (aHUS) of C3 glomerulopathie (C3G), en 697 LMD patiënten. De locatie waar een genetische variant zich bevindt bepaalt waar en welk effect deze heeft op het eiwit. Men kan ontrafelen hoe een eiwit werkt door vast te stellen welke delen (domeinen) van een eiwit functioneel worden aangetast door genetische varianten. Dergelijk inzicht kan helpen begrijpen hoe het complementsysteem verantwoordelijk is voor twee heel verschillende ziektebeelden. Bij patiënten met nierziekten werd een hogere frequentie van eiwit veranderende varianten

gevonden in SCR20 van FH en in het serine protease domein van FI. Voor patiënten met LMD bevatte de N-terminus van FH, in het bijzonder de SCR3, SCR5 en SCR7 domeinen, meer eiwit veranderende varianten, naast het SRCR domein in FI en MG3 domein van C3. Dit bevestigt eerder onderzoek dat aantoonde dat FH belangrijk is voor de binding aan het nier endotheel via de C-terminus, terwijl de N-terminus van FH betrokken is bij C3b-regulatie in het oog. Veranderingen in het SRCR domein van FI hebben waarschijnlijk invloed op de expressie en dus de algehele activiteit, terwijl nier-gerelateerde FI veranderingen betrokken zijn bij structurele elementen van het eiwit. Veranderingen in het MG3 domein van C3 kunnen de interactie met FH verstoren en de afbraak van C3b belemmeren. Samengenomen zagen we een grote overlap van varianten bij aHUS/C3G en LMD, maar was een duidelijke clustering van varianten binnen specifieke domeinen.

In **hoofdstuk 9** hebben we whole-exome sequencing informatie van 1125 LMD patiënten en 1361 controles geanalyseerd met behulp van een 'single variant' analyse. Hiermee bevestigden we de associatie van veelvoorkomende varianten in *CFH* en *ARMS2*. Verder gebruikten we een 'gene-based burdentest', genaamd 'combined multivariate and collapsing' (CMC), die de associatie van meerdere zeldzame varianten per gen samenvoegt. Met behulp van de CMC test hebben we een 'burden' waargenomen voor het *COL8A1* gen, veroorzaakt door 14 zeldzame varianten die het eiwit veranderen. Aankleuring van retina cellen van muizen toonde aan dat het COL8A1 eiwit zich in het membraan van Bruch bevindt. Onze bevindingen suggereren dat eiwit veranderende varianten in COL8A1 de werking van het membraan van Bruch kunnen veranderen, en daarmee bijdragen aan de ophoping van drusen en de ontwikkeling van LMD.

In **conclusie**, een voorspelling doen over de werking van nieuw ontdekte genetische varianten is een uitdaging, zelfs als deze varianten voorkomen in genen die al eerder onderzocht zijn in relatie tot LMD. In dit proefschrift hebben we beschreven hoe we zeldzame genetische varianten hebben geïdentificeerd in genen die een rol spelen in het afweersysteem, in de steunende weefselstructuur (de extracellulaire matrix), en in erfelijke macula dystrofieën. Uiteindelijk bleek aanvullende informatie vanuit biologische experimenten noodzakelijk om het effect van de genetische varianten goed te kunnen beoordelen.

Inzicht in de functionele gevolgen van deze varianten helpt om het onderliggende ziektemechanisme te begrijpen. Een beter begrip van het ziektemechanisme is nodig om de behandelmogelijkheden te optimaliseren. Kennis over de rol van zeldzame genetische varianten kan ons helpen om in de toekomst effectievere behandelingen en persoonsgerichte zorg voor LMD te ontwikkelen.

LIST OF PUBLICATIONS

M. J. Geerlings, E. B. Volokhina, N. van de Kar, J. Corominas, M. Pauper, C. B. Hoyng, E. K. de Jong, L. P. van den Heuvel, and A. I. den Hollander. "Genotype-phenotype correlations of low frequency genetic variants in the complement system in renal disease and age-related macular degeneration" *submitted*

M. Kremlitzka, **M. J. Geerlings,** S. de Jong, B. Bakker, S.C. Nilsson, S. Fauser, C. B. Hoyng, E. K. de Jong, A. I. den Hollander, and A. M. Blom. "Functional Analysis of Rare-Genetic Variants in Ccomplement Component C9 in Patients with Age-Related Macular Degeneration" *in preparation*

E. Kersten, **M. J. Geerlings**, M. Pauper, E. K. de Jong, C. C. W. Klaver, A. I. den Hollander, and C. B. Hoyng. "Genetic screening for macular dystrophies in patients clinically diagnosed with dry age-related macular degeneration " *in preparation*

M. J. Geerlings, E. Kersten, J. M. M. Groenewoud, L. G. Fritsche, C. B. Hoyng, E. K. de Jong, and A. I. den Hollander. "Geographical Distribution of Rare Variants Which Are Associated with Age-Related Macular Degeneration" *Molecular Vision, in press*

J. Corominas, J. M. Colijn, **M. J. Geerlings**, M. Pauper, B. Bakker, N. Amin, L. Lorés-Motta, E. Kersten, A. Garanto, J. A. M. Verlouw, J. G. J. van Rooij, R. Kraaij, P. T. V. M. de Jong, A. Hofman, J. R. Vingerling, T. Schick, S. Fauser, E. K. de Jong, C. M. van Duijn, C. B. Hoyng, C. C. W. Klaver, and A. I. den Hollander. "Whole-exome sequencing in age-related macular degeneration identifies rare protein-altering variants in COL8A1, a component of Bruch's membrane" *submitted*

L. Lorés-Motta, C. C. Paun, J. Corominas, M. Pauper. **M. J. Geerlings**, L. Altay, T. Schick, M. R. Daha, S. Fauser, C. B. Hoyng, A. I. den Hollander, and E. K. de Jong. "GWAS reveals variants in CFH and CFHR4 associated with systemic complement activation: implications in age-related macular degeneration" *Ophthalmology, in press*

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CURRICULUM VITAE

Martina (Maartje) Johanna Geerlings was born on the 8th of November 1989 in Zwolle, the Netherlands. In 2007, she graduated from Bilingual Higher General Secondary Education at the Van der Capellen Scholengemeenschap, in Zwolle and received a English degree (higher level) from the international baccalaureate organization. She started the bachelor of applied sciences in Biotechnology with a specialization in Forensic sciences at Van Hall Larenstein in Leeuwarden, the Netherlands. During her internships at Utrecht University Medical Center at the departments of diagnostic pathology and heart transplantation group she developped an interest in biomedical sciences.

In 2011, after obtaining her bachelor degree she started with the master of Molecular Life Sciences with the specialization in Biomedical research at Wageningen University and research center, Wageningen, the Netherlands. For her master thesis she worked in the division of Biomedical genetics of Utrecht University Medical Center under the supervision of Gijs van Haaften. Here, Maartje used a personalized sequencing chip to elucidate the genetic background of human obesity. Afterwards, she went to the Institute of Child Health of University College London, London, United-Kingdom to the group of Phil Beales. Here, Maartje tested the effect of drugs on neural crest cell development in zebrafish. Furthermore, she used the CRISPR-Cas9 system to disrupt gene function in the zebrafish model system.

Upon completion of her master thesis in October 2013, Maartje started her PhD project under the supervision of professor Anneke den Hollander, professor Carel Hoyng and doctor Eiko de Jong at the department of Ophthalmology at Radboud university medical center in Nijmegen, the Netherlands. Her four years of doctoral research are presented in this thesis entitled "Studies of rare genetic variants in age-related macular degeneration" and was defended on februari 15th, 2018. Completing her doctoral studies, she resides in Nijmegen and hopes to continue, in the next step of her carreer, to use her knowledge on genetic analyses to improve patient care.

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Lieve **Julia**, soms zijn wij samen als water en vuur, stiekem denk ik dat dit komt omdat we in de meeste aspecten sterk op elkaar lijken. Gedreven, perfectionistisch en altijd het beste van jezelf en anderen verwachten. Jouw prachtige ontwerp is door iedereen te bewonderen op de kaft van dit proefschrift.

Lieve **Papa** en **Mama**, bedankt voor jullie eeuwige steun in mijn ambitieuze loopbaankeuzes. Jullie hebben mij geleerd om door te zetten, mijn dromen achterna te gaan en mij nooit naar beneden te laten praten door anderen. Liefde voor uitgebreid koken, bakken en eten (het liefst in combinatie met een goed glas wijn) hebben jullie aan mij doorgegeven. Gezellig samen eten en drinken met goede vrienden en familie "zo moet het leven zijn".

Allerliefste **Erik**, wie had gedacht dat 'naar rechts swipen' zo goed uit zou pakken? Jij hebt mij door de laatste stressvolle maanden heen geholpen met een luisterend oor en vele knuffels. Ik ben dankbaar dat ik mij nooit heb hoeven verantwoorden betreft mijn lange dagen of als het huishouden er even bij in schoot. Ik waardeer het enorm dat je blijft proberen mij te begrijpen als ik weer een verhaal afsteek over mijn onderzoek, zelfs als ik in mijn enthousiasme vergeet de termen uit te leggen. Stiekem weet ik dat 'AMD' voor jou nog steeds Arbo en Milieu Dienst betekend. Lieverd, eindelijk is het zo ver: het is tijd om onze rugzakken in te pakken!

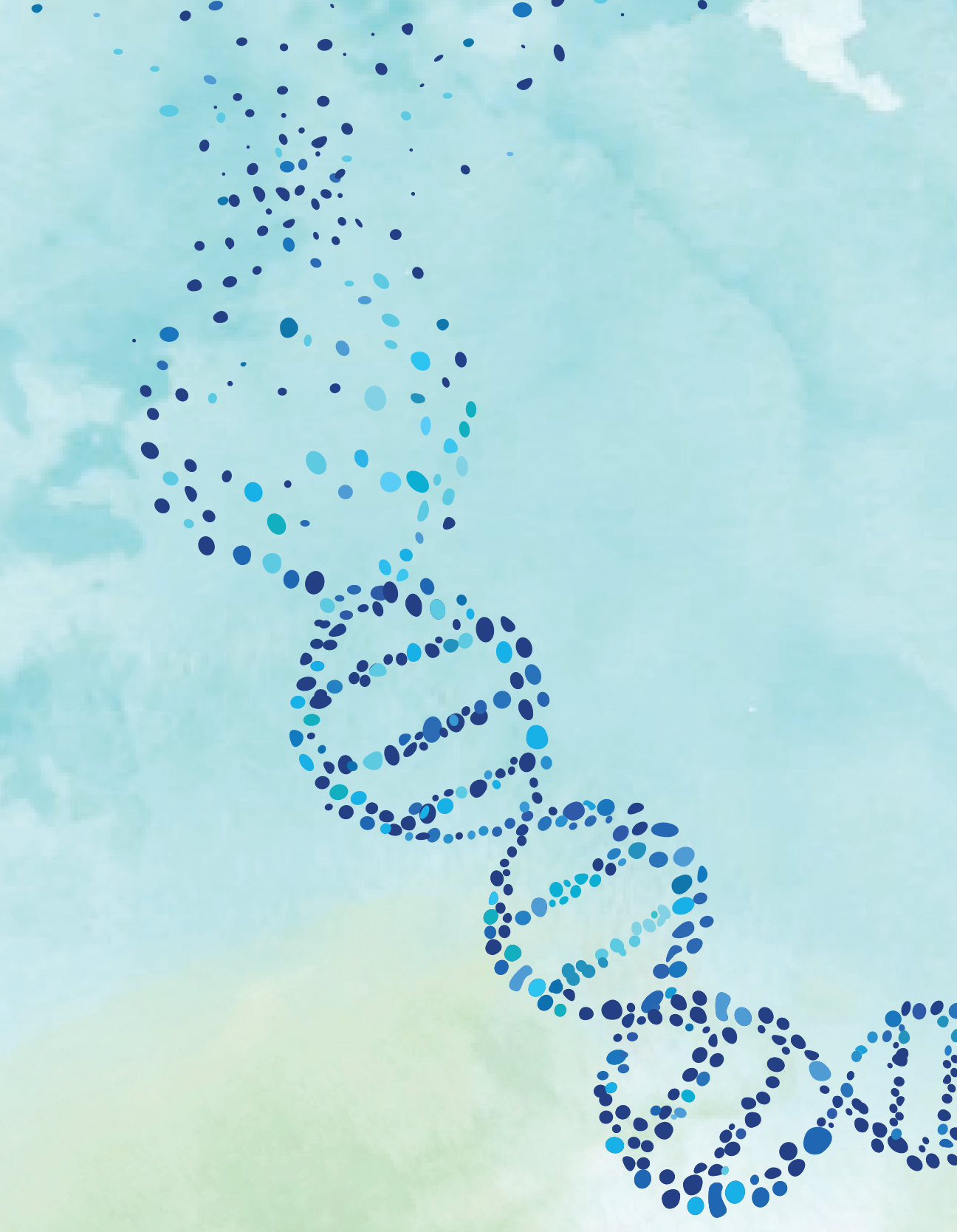
PHD PORTFOLIO

Maartje J. Geerlings
Department of Ophthalmology
Radboud Institute for Molecular Life Sciences

PhD period: 01-10-2013 to 01-10-2017
Promotors: Prof. dr. Anneke I. den Hollander
Prof. dr. Carel B. Hoyng
Co-promotor: Dr. Eiko K. de Jong

	Year(s)	ECTS
TRAINING ACTIVITIES		
a) *Courses & Workshops*		
- *Graduate Course*	2014	2.00
- *Genetic Association workshop*	2014	1.75
- *Opfriscursus statistiek voor promovendi*	2014	1.00
- *Novel Therapies for Retinal dystrophies*	2014	0.30
- *Genetic Epidemiology Course (MED-5E005)*	2014	7.00
- *Scientific Integrity for PhD students*	2015	1.00
- *The Art of Presenting Science*	2015	1.50
- *Academic writing & how to write a medical scientific paper*	2015/2016	3.20
- *Intermediate & advanced workshop in UCSC*	2015	0.40
- *Basiscursus Regelgeving en Organisatie voor Klinisch onderzoeker*	2016	1.25
- *MBSR: Mindfulness Based Stress Reduction for PhD students*	2017	2.00
- *Mindfulness: compassie*	2017	1.50
b) *Seminars & lectures*		
- *RIMLS and other lectures (n=13)*	2013-2017	1.30
- *RIMLS Technical forms (n=3)*	2014	0.30
- *Radboud Research Rounds (n=2)*	2014-2017	0.20
- *RIMLS PhD retreat* (n=4)*	2014-2017	2.50
- *Sensory Disease Meeting (weekly; 12 presentations*)*	2013-2017	4.00
- *Journal Club Human Genetics (weekly; 3 presentations*)*	2013-2016	3.00
- *Theme Discussion Human Genetics (weekly; 3 presentations*)*	2014-2017	3.00
- *Grant writing workshops and career development (n=4)*	2017	1.70
c) *Symposia & congresses*		
- *RIMLS New Frontiers Symposium (n=3)*	2013-2015	3.00
- *Refereeravonden Oogheelkunde (n=2)*	2014-2015	0.20
- *EUGENDA retreat & AMD-day *** (n=3)*	2013-2017	1.50
- *Dutch Ophthalmology PhD Students conference ** (n=3)*	2014-2017	2.00
- *Publieksavond Therapie in Zicht*	2015	0.10
- *Annual ARVO 2015 conference**	2015	1.75
- *Young researcher visioncamp#*	2015	1.25
- *6th International NOG-Symposium on AMD#*	2015	1.00
- *ASHG 2016 conference#*	2016	1.50
- *Nederlands Oogheelkunde Gezelschap (NOG): bijeenkomst**	2017	0.50
TEACHING ACTIVITIES		
d) *Lecturing and Supervision of internships / other*		
- *Mini-internship 3rd year medical students*	2014	1.30
- *Mini-internship 1st year biomedical students*	2014	1.00
- *Writing and distribution patient newsletter*	2015	0.50
- *Information distribution to patients at the '50plusBeurs'*	2015	0.50
TOTAL		**55.0**

* oral presentation; # poster presentation



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